IOWA STATE COLLEGE

# JOURNAL OF SCIENCE

**Published on the first day of October, January, April, and July**

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All remittances should be addressed to Collegiate Press, Inc., Iowa State College, Ames, Iowa.

Single Copies: One Dollar. Annual Subscription: Three Dollars; in Canada, Three Dollars and Twenty-five Cents; Foreign, Three Dollars and Fifty Cents.

Entered as second-class matter January 16, 1935, at the postoffice at Ames, Iowa, under the act of March 3, 1879.

# A FUNCTIONAL METHOD FOR THE SOLUTION OF THIN PLATE PROBLEMS APPLIED TO A SQUARE, CLAMPED PLATE WITH A CENTRAL POINT LOAD

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The present paper employs a new point of view for obtaining approximate solutions of linear differential equations. In this method the approximations are determined by the use of functionals. A person experienced in the approximate solution of such differential equations will recognize that the results obtained by the use of functionals are, at times, the equivalent of results obtained by the use of other approximation methods. As a matter of fact, it will be found that the functional method welds most of the standard methods, and many more, into a coherent whole.

While the particular problem to which we shall apply this method has been solved using other points of view, the present solution illustrates the versatility of the functional idea. In particular, this work illustrates how solutions are obtained by this method when no functions which satisfy either the boundary conditions or the differental equations are known.

The general method as first enunciated by Gross[1,2] is as follows: We desire a solution to the linear operational equation

$$L(w) = f \tag{1}$$

and the $b$ linear boundary conditions

$$B_l(w) = g \text{ when } \lambda_l = 0 \qquad (l = 1, 2, \ldots, b). \tag{2}$$

in which

$$\lambda_l \equiv \lambda_l(x_1, x_2, \ldots, x_n) = 0 \tag{4}$$

are the equations of the boundary.

It is assumed that $w$ can be represented by a sum of expansion functions,

$$w = a_i\phi_i, \tag{5}$$

where the $a_i$'s are constants to be determined and the $\phi_i$'s are known functions. A subscript, repeated in a term, has the usual meaning that the term is to be summed for all values of the subscript, *e. g.*

$$a_i\phi_i = \sum_{i=0}^{\infty} a_i\phi_i$$

unless the contrary is expressly stated. If the $\phi_i$'s constitute a complete

set of functions, it is known that a very wide range of functions can be expressed by such a sum. It is assumed that a finite number of them will be an approximation $w^k$ to the solution $w$.

$$w \cong w^k = a^k{}_m \phi_m \, (m = 0, 1, 2, \ldots, k) \tag{7}$$

Substitution of the finite expansion (7) for $w$ in equations (1) and (2) gives

$$L(a^k{}_m \, \phi_m) \cong f$$

or

$$a^k{}_m \, L(\phi_m) - f \cong 0 \tag{8}$$

and

$$a^k{}_m \, B_l(\phi_m) \cong g \text{ on the boundary} \tag{9}$$

since the linear operators commute the $a^k{}_m$'s.

Suppose we now choose two functional families $F_j$ and $G_j$ which are linear. The $F$'s are defined in the region $S$ of the plate; the $G$'s on $s$, the boundary of the plate. The approximation defined by the functional families $F_j$ and $G_j$ and the functions of expansion, $\phi_m$ is determined when we require that the coefficients, $a^k{}_m$ satisfy the equations:

$$F_j[a^k{}_m L(\phi_m) - f] = 0 \, (j = 0,1, \ldots, k-r); \tag{10}$$

$$G_j[a^k{}_m \, B_l(\phi_m) - g] = 0 \, (j = k - r, \ldots, k). \tag{11}$$

We have thus reduced the problem of finding an approximate solution of the differential equation to the problem of finding a solution to an arbitrary number of linear equations in the $a^k{}_m$'s. The number of equations to be solved is arbitrary because it depends upon the number of terms in the assumed approximation function. The resultant linear equations in the $a^k{}_m$'s are then solved for the $a^k{}_m$'s and an approximation for $w$ results.

Thus a given set of functionals and functions define an approximation to the solution of the problem. The properties of these approximations, such as their limits of error, have not been entirely determined[2], partly because of the complete generality of the method.

The choice of the equations for the determination of the $a^k{}_m$'s is usually guided by the relative degrees of approximation desired for the differential equation (1) and the boundary conditions (2). In general, different choices of equations for the determination of the $a^k{}_m$'s leads to different approximations to $w$. For an approximation with more terms, equations (10) and (11) with more terms in $a^k{}_m \phi_m$ and hence more, and usually different equations in the $a^k{}_m$'s are to be solved. In general, the resultant $a^k{}_m$'s will be different from different approximations. If the $a^k{}_m$'s remain the same such approximations may be called stabile, e.g. Fourier series.

It is easily seen that most of the standard methods for determining approximate solutions are particular examples of this functional method.

If the boundary conditions are homogeneous we may be able to choose expansion functions so that each (and hence their sum) satisfies these conditions. Suppose we choose to satisfy the differential equation by application of the functional family

$$F_j[\ ] \equiv \int \phi_m [\quad] d\tau \tag{12}$$

to (8). There rersults

$$a^k{}_m \int \phi_m L(\phi_m)\, d\tau = \int \phi_m f\, d\tau \tag{13}$$

and we have the same equations as are given by the Ritz[3] method. Suppose we choose to satisfy the differential equation by employing the functional family

$$F_j[\ ] \equiv \int L(\phi_m)[\quad] d\tau. \tag{14}$$

There results

$$a^k{}_m \int [L(\phi_m)]^2 d\tau = \int f L(\phi_m)\, d\tau \tag{15}$$

which are the equations defining the Bousinesq[4] or "least square" approximation.

If our problem is of such a nature that $w = f(s)$ on $I$ and $\dfrac{\partial w}{\partial \nu} = g(s)$ on $II$, we may be able to choose the expansion functions such that each satisfies the differential equation and their sum satisfies $\dfrac{\partial w}{\partial \nu} = g(s)$ on $II$. If we apply the functional family

$$F_j \equiv \int_I \left[\quad\right] \frac{\partial \phi_j}{\partial \nu} ds \tag{16}$$

to the second boundary condition there results

$$\int_I \left[ a^k{}_m \phi_m - f(s) \right] \frac{\partial \phi_j}{\partial \nu} ds = 0 \tag{17}$$

These are the same equations as were determined by Trefftz[5].

Gross[1 2] has included many other well known mathematical methods and procedures as special cases of the functional method. This includes:

1. Perturbation theory.
2. Taylor's series expansion.
3. Expansion in orthogonal functions.
4. Certain solutions of integral equations.
5. Method of Krawtchouk.

## Homogeneous, Square, Clamped Plate with Center Point Load

*The Problem*

To solve this problem it is necessary to obtain a function $w$, representing the deflection of the plate due to the point load $P$ at the origin of coordinates, which satisfies the differential equation

$$\nabla^4 w = \frac{P}{N} = 0, \quad \nabla^4 = \frac{\partial^4}{\partial x^4} + 2\frac{\partial^4}{\partial x^2 \partial y^2} + \frac{\partial^4}{\partial y^4} . \tag{18}$$

The coordinate plane is the plane of the middle surface of the undeflected plate with the origin at the centroid of the plate.

Because the plate is clamped,

$$\frac{\partial w}{\partial \nu} = 0 \qquad \text{for} \quad \begin{matrix} x = \pm a \\ y = \pm a \end{matrix}, \tag{19}$$

$$w = 0 \qquad \text{for} \quad \begin{matrix} x = \pm a \\ y = \pm a \end{matrix} \tag{20}$$

where $a$ is half the distance between successive corners of the plate. Equation (19) is equivalent to the two equations

$$\frac{\partial w}{\partial x} = 0 \qquad \text{for} \quad x = \pm a ,$$

$$\frac{\partial w}{\partial y} = 0 \qquad \text{for} \quad y = \pm a . \tag{21}$$

Because $\nabla^4 w = \dfrac{p}{N}$ we have the associated condition that the derivatives must possess discontinuities of a type such that the shear integral has the value

$$\int \left[ N \frac{\partial \nabla^2 w}{\partial \nu} \right] d\theta = -P . \tag{22}$$

*Application of Theory*

For this problem we will use the simple functionals defined as follows:

$$F_k[f(x, y)] \equiv f(x, y) \Big|_{\substack{x = x_k \\ y = y_k}} \tag{23}$$

This simply means that the function $f(x, y)$ is reduced to its value at the point $(x_k, y_k)$, obtained by setting $x = x_k$ and $y = y_k$ in $f(x, y)$. The constants of the sum of expansion functions will be determined by evaluating

that solution at the same number of points as there are constants to be determined.

The expansion function chosen was

$$w^k = A_o(x, y) + R^k(x, y) \tag{24}$$

where $R^k(x, y)$ is a polynomial in $x$ and $y$, and $A_o$ is a function satisfying the associated condition (22). The usual function for this purpose is

$$A_o(x, y) = \frac{Pr^2}{8\pi N} \log_e \frac{a}{r} \tag{25}$$

where $r = \sqrt{x^2+y^2}$ and $N = E'I$, $E' = \dfrac{E}{1-\sigma^2}$ and $I$ is the moment of inertia of a unit cross section of area of the plate about the axis where it intersects the $x, y$ plane. As usual $E$ is Young's modulus and $\sigma$ is Poisson's ratio. It is to be observed that the choice of expansion functions will greatly influence the rapidity of convergence of $w^k$.

Because of the symmetry conditions of the plate

$$R^k(x, y) = R^k(-x, y) = R^k(x,-y) = R^k(-x,-y). \tag{26}$$

This makes it necessary that we include only even powers of $x$ and $y$. Also,

$$R^k(x, y) = R^k(y, x). \tag{27}$$

This places the further restriction upon $R^k(x, y)$ that the coefficient of $x^\alpha y^\beta$ equal the coefficient of $x^\beta y^\alpha$. With these conditions in mind we have chosen $R^k$ a sum of homogeneous polynomials and

$$\begin{aligned} w^k = \frac{P(x^2+y^2)}{8\pi N} \log_e \frac{a}{r} &+ A_1 + A_2(x^2+y^2) + A_3(x^4+y^4) \\ &+ A_4x^2y^2 + A_5(x^6+y^6) + A_6(x^2y^4+x^4y^2) \\ &+ A_7x^4y^4 + A_8(x^2y^6+x^6y^2) + A_9(x_8+y_8) \\ &+ A_{10}(x^{10}+y^{10}) + A_{11}(x^8y^2+x^2y^8) + A_{12}(x^6y^4+x^4y^6). \end{aligned} \tag{28}$$

If the functional (23) is now applied to the equations (18), (20) and (21), with $w^k$ introduced, thus reducing $x$ and $y$ to their values at various points throughout the plane, we can solve the resultant equations for the coefficients in $R^k(x, y)$. We need only one of the slope equations (20) because of the symmetry of the problem. The points chosen are shown on the diagram of the plate (Figure 1); the resultant equations are equations (29) - (40).

The points represented by a dot are the points actually used and the points represented by a cross are those included by symmetry. The

points marked 3 were used in all three equations: normal derivative, displacement, and differential equation.

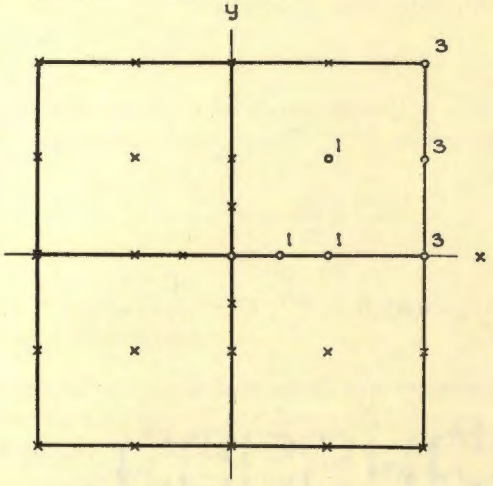


Figure 1

The boundary condition equations are:

$x = a\ y = 0,\ w = 0,$

$$0 = A_1 + A_2a^2 + A_3a^4 + A_5a^6 + A_9a^8 + A_{20}a^{20}; \tag{29}$$

$x = a,\ y = a/2,\ w = 0,$

$$0 = \frac{5/4Pa^2}{8\pi N}\log_e\frac{2}{\sqrt{5}} + A_1 + \frac{5}{4}A_2a^2 + \frac{17}{16}A_3a^4 + \frac{1}{4}A_4a^4 + \frac{65}{64}A_5a^6 + \frac{5}{16}A_6a^6 + \frac{1}{16}A_7a^8 + \frac{17}{64}A_8a^8 + \frac{257}{256}A_9a^8 + \frac{1025}{1024}A_{10}a^{10} + \frac{65}{256}A_{11}a^{10} + \frac{5}{64}A_{12}a^{10}; \tag{30}$$

$x = a,\ y = a,\ w = 0,$

$$0 = \frac{2Pa^2}{8\pi N}\log_e\frac{1}{\sqrt{2}} + A_1 + 2A_2a^2 + 2A_3a^4 + A_4a^4 + 2A_5a^6 + 2A_6a^6 + A_7a^8 + 2A_8a^8 + 2A_9a^8 + 2A_{10}a^{10} + 2A_{11}a^{10} + 2A_{12}a^{10}; \tag{31}$$

$$x = a,\ y = 0,\ \frac{\partial w}{\partial v} = 0,$$

$$0 = -\frac{Pa}{8\pi N} + 2A_2a + 4A_3a^3 + 6A_5a^5 + 8A_9a^7 + A_{10}a^{10}; \tag{32}$$

$$x = a,\ y = \frac{a}{2},\ \frac{\partial w}{\partial x} = 0,$$

$$\begin{aligned} 0 = \frac{Pa}{8\pi N}\left[-1 + 2\log_e \frac{2}{\sqrt{5}}\right] &+ 2A_2a + 4A_3a^3 + \tfrac{1}{2}A_4a^3 \\ &+ 6A_5a^5 + \frac{9}{8}A_6a^5 + \frac{1}{4}A_7a^7 + \frac{49}{32}A_8a^7 + 8A_9a^7 \\ &+ 10A_{10}a^9 + \frac{257}{128}A_{11}a^9 + \frac{7}{16}A_{12}a^9; \end{aligned} \tag{33}$$

$$x = a,\ y = a,\ \frac{\partial w}{\partial x} = 0,$$

$$\begin{aligned} 0 = \frac{Pa}{8\pi N}\left[-1 + 2\log_e \frac{1}{\sqrt{2}}\right] &+ 2A_2a + 4A_3a^3 + 2A_4a^3 \\ &+ 6A_5a^5 + 6A_6a^5 + 4A_7a^7 + 8A_8a^7 + 8A_9a^7 \\ &+ 10A_{10}a^9 + 10A_{11}a^9 + 10A_{12}a^9; \end{aligned} \tag{34}$$

The differential equation conditions are:

$$x = a,\ y = 0,\ \nabla^4 w = 0,$$

$$\begin{aligned} 0 = 8A_4 &+ 48A_3 + 360A_5a^2 + 72A_6a^2 + 24A_7a^4 \\ &+ 120A_8a^4 + 224A_{11}a^6 + 1680A_9a^4 \\ &+ 5040A_{10}a^6 + 24A_{12}a^6; \end{aligned} \tag{35}$$

$$x = a,\ y = {}^a/_2,\ \nabla^4 w = 0,$$

$$\begin{aligned} 0 = 8A_4 &+ 48A_3 + 450A_5a^2 + 90A_6a^2 + \frac{195}{2}A_7a^4 \\ &+ \frac{615}{2}A_8a^4 + \frac{1505}{2}A_{11}a^6 + 1785\,A_9a^4 \\ &+ \frac{20475}{4}A_{10}a^6 + \frac{2895}{8}A_{12}a^6; \end{aligned} \tag{36}$$

$$x = a,\ y = a,\ \nabla^4 w = 0,$$

$$0 = 8A_4 + 48A_3 + 720A_5a^2 + 144A_6a^2 + 336A_7a^4 + 960A_8a^4 + 3808A_{11}a^6 + 3360A_9a^4 + 10080A_{10}a^6 + 2208A_{12}a^6; \tag{37}$$

$$x = a/2,\ y = a/2,\ \nabla^4 w = 0,$$

$$0 = 8A_4 + 48A_3 + 180A_5a^2 + 36A_6a^2 + 21A_7a^4 + 60A_8a^4 + \frac{119}{2}A_{11}a^6 + 210A_9a_4 + \frac{315}{2}A_{10}a^6 + \frac{69}{2}A_{12}a^6; \tag{38}$$

$$x = a/2,\ y = 0,\ \nabla^4 w = 0,$$

$$0 = 8A_4 + 48A_3 + 90A_5a^2 + 18A_6a^2 + \frac{3}{2}A_7a^4 + \frac{15}{2}A_8a^4 + \frac{7}{2}A_{11}a^6 + 105A_9a^4 + \frac{315}{4}A_{10}a^6 + \frac{3}{8}A_{10}a^6; \tag{39}$$

$$x = a/4,\ y = 0,\ \nabla^4 w = 0,$$

$$0 = 8A_4 + 48A_3 + \frac{45}{2}A_5a^2 + \frac{9}{2}A_6a^2 + \frac{3}{32}A_7a^4 + \frac{15}{32}A_8a^4 + \frac{7}{128}A_{11}a^6 + \frac{105}{16}A_9a^4 + \frac{315}{256}A_{10}a^6 + \frac{3}{512}A_{12}a^6. \tag{40}$$

*The Solution*

The equations (29)-(40) are linear equations in the parameters $A_1$, $A_2$, $A_3$, .... These can be solved by the ordinary methods. The work of computation may be checked by substituting the calculated values of the parameters $A_1$, $A_2$, $A_3$, ...., as derived. This shows that these constants are accurate to six places.

When these values are substituted in equation (28) there results the following approximation for $w$:

$$\frac{8\pi N}{P} w^k = (x^2+y^2)\log_e \frac{a}{r} - .562487a^2 + .556212(x^2+y^2)$$
$$+ .075704\frac{(x^4+y^4)}{a^2} - .454207\frac{x^2y^2}{a^2}$$
$$- .065438\frac{(x^6+y^6)}{a^4} + .327202\frac{(x^2y^4+x^4y^2)}{a^4}$$
$$+ .024818\frac{x^4y^4}{a^6} - .009927\frac{(x^2y^6+x^6y^2)}{a^6}$$
$$+ .000352\frac{(x^8+y^8)}{a^6} - .002543\frac{x^{10}+y^{10}}{a^8}$$
$$+ .068670\frac{(x^8y^2+x^2y^8)}{a^8} - .106820\frac{x^6y^4+x^4y^6}{a^8}.$$

The ratio of the maximum value of $w^k$ on the boundary to the center deflection is .3%; of the normal derivative $3/a\%$. A homogeneous biharmonic polynomial has certain fixed ratios between its coefficients, hence, we can see how well the differential equation is satisfied by finding these ratios. These ratios, exact and as found from our solution are given in Table 1.

TABLE 1. *Ratios of the coefficients of the biharmonic polynomials.*

| Ratio | Exact values | As found from Approximation function |
|---|---|---|
| $A_4/A_3$ | — 6.00000 | — 5.99979 |
| $A_6/A_5$ | — 5.00000 | — 5.00017 |
| $A_8/A_5$ | —28.0000 | —28.2358 |
| $A_7/A_5$ | 70.0000 | 70.5916 |
| $A_{11}/A_{10}$ | —27.0000 | —27.0084 |
| $A_{12}/A_{10}$ | 42.0000 | 42.0131 |

Evaluation of the shear integral

$$8\int_0^a \left[N\frac{\partial\nabla^2 w}{\partial x}\right]dy = -P \tag{40}$$

around the boundary of the plate should give us $-P$. It actually gives

$$= (1.000027)P \tag{41}$$

The maximum deflection (the value of $w$ at (0,0)) from equation (39) is

$$.022452Pa^2/N \tag{42}$$

J. Barta[6] obtained by the Trefftz method

$$.0224Pa^2/N. \tag{43}$$

H. Marcus[7] obtained by difference equations

$$.02297Pa^2/N. \tag{44}$$

G. Pickett[8] obtained by energy minimization

$$.02153Pa^2/N. \tag{45}$$

The maximum bending moment is

$$-.125679P; \tag{46}$$

this is slightly dependent upon $\sigma$ since $w|_{y=a}$ is small in value but not zero. The moment was given above for $\sigma = .3$.

S. Timoshenko[9] obtained by superposition

$$-.1257P. \tag{47}$$

D. Young[10] obtained by a similar method

$$-.1257P. \tag{48}$$

G. Pickett obtained by energy minimization

$$-.1363P. \tag{49}$$

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# PRELIMINARY STUDIES ON THE USE OF DINITRO-O-CRESOL DUSTS IN GRASSHOPPER CONTROL[1]

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Received May 17, 1940

The experimental use of proprietary compounds as contact insecticides to destroy mass-formations of locust and "tucuras" (grasshoppers) in the Republic of Argentina aroused the interest of the authors and suggested the desirability of testing certain nitro-cresols and related compounds on grasshoppers in Iowa.

Chemical analyses[2] of the two dusts most widely used in Argentina were as follows:

> "A-Dust"—Active material 14 per cent, consisting entirely of 3,5-dinitro-o-cresol; inactive material 86 per cent, diatomaceous earth.
>
> "B-Dust"—Active material 12 per cent, a mixture of 3,5-dinitro-o-cresol, some 5-nitro-o-cresol and sodium salts of these two compounds; inactive material 88 per cent, consisting of diatomaceous earth.

The use of dinitro-o-cresol compounds as insecticides is not new. As early as 1893 Lodeman (1893) imported antinonnin, a potassium dinitrocresylate compound, from Germany and reported that as a spray it was highly caustic on some fruit trees and that it did not control plant lice.

A rather extensive investigation on the relative toxicity of a long series of nitro-phenols and related compounds reported by Tattersfield, Gimingham and Morris (1925), stimulated interest in this field. This paper was followed by other papers from the same and other workers who have studied the use of dinitro compounds as contact sprays and particularly as ovicides. Kagy and Richardson (1936) published a brief review of the older literature in their paper which introduced the new compound, 2,4-dinitro-6-cyclohexylphenol, now receiving considerable study in several states.

About 1935, German entomologists began to experiment with dinitro-o-cresol and related compounds as contact dusts. Marcus (1937), Thiem (1938), Schwerdtfeler (1939) and Hofman (1940) have made important contributions on the subject and all cite the works of their contemporaries.

Twenty-five chemical compounds of technical grade or better obtained from reliable chemical houses were used in the preliminary experiments. In these tests, 50 adults of *Melanoplus bivittatus* Say (25 males

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[1] Journal Paper No. J-753 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 649.

[2] The writers are greatly indebted to Mr. A. Goytea, Sub-Director Defensa Agricola, Ministerio de Agricultura, Buenos Aires, for kindly supplying the authors with a supply of the two dust chemicals.

and 25 females) were individually dipped in finely ground dust of the various chemicals and then placed in screened cages for observation. The average time elapsing between dusting and death for each of the compounds is given in table 1.

The four most toxic compounds used in the preliminary series were then subjected to further tests. In these experiments, dust mixtures con-

TABLE 1. *Average time elapsing between treatment and death of Melanoplus bivittatus adults when thoroughly coated with dust of various chemical compounds*

| Chemical compound (dust) | Time required to kill (minutes) | | | |
|---|---|---|---|---|
| | Males | | Females | |
| | Range | Mean* | Range | Mean* |
| 3,5-Dinitro-o-cresol CP. | 8 - 20 | 14 | 14 - 29 | 21 |
| 3,5-Dinitro-o-cresol T. | 9 - 18 | 15 | 16 - 30 | 22 |
| 2,4-Dinitro phenol | 8 - 19 | 14 | 17 - 34 | 22 |
| Sodium arsenite | 14 - 43 | 32 | 18 - 54 | 35 |
| 2,4-Dinitro-o-cyclohexyl phenol | 22 - 42 | 33 | 52 - 97 | 71 |
| 3,5-Dinitro-o-cresol, sodium salt | 28 - 76 | 49 | 54 - 121 | 71 |
| 2,6-Dichloronitrophenol | 48 - 73 | 59 | 55 - 116 | 81 |
| 2,5-Dichloronitrobenzene | 46 - 316 | 182 | 84 - 253 | 168 |
| Hexochlorphenol | 125 - 156 | 135 | 83 - 341 | 200 |
| O-Nitroaniline | 50 - 155 | 103 | 56 - 447 | 202 |
| Sodium dinitro-6-cyclohexylphenate | 69 - 316 | 135 | 89 - 393 | 210 |
| 2,4-Dinitroanisole | 162 - 383 | 288 | 261 - 413 | 330 |
| P-Nitrochlorobenzene | 150 - 233 | 185 | 143 - 690 | 358 |
| P-Nitrophenetole | 154 - 289 | 149 | 187 - 637 | 366 |
| P-Nitrotoluene | 68 - 454 | 310 | 304 - 731 | 440 |
| M-Dinitrobenzene | 138 - 483 | 272 | 380 - 1440+ | 510 |
| 2,4-Dinitrotoluene | 90 - 395 | 240 | 306 - 1440+ | 720+ |
| 3,5-Dinitrobenzoic acid | 192 - 667 | 330 | 286 - 1440+ | 720+ |
| Picric acid | 110 - 697 | 430 | 278 - 1440+ | 720+ |
| 2,4-Dinitrophenylhydrazine | 68 - 720+ | 720+ | 468 - 1440+ | 720+ |
| P-Nitrodimethylaniline | 163 - 720+ | 1440+ | 720+ - 1440+ | 720+ |
| Dinitronaphthaline | 1440+ | 1440+ | 1440+ | 1440+ |
| 3,4-Dichloronitrobenzene | 1440 | 1440+ | 1440+ | 1440+ |
| 2,4-Dinitroaniline | 1440 | 1440+ | 1440+ | 1440+ |
| 2,6-Dichloronitroaniline | 1440 | 1440+ | 1440+ | 1440+ |

*Each mean obtained from 25 grasshoppers treated and observed individually.

taining definite percentages of the chemicals in diatomaceous earth were prepared and adult 'hoppers were individually dipped in the different dust-mixtures. At least 50 adults of *M. bivittatus* Say (25 males and 25 females) were used in each test. The results of these experiments are given in table 2. As 3,5-dinitro-o-cresol continued to stand out as the most toxic compound, it was selected for further study.

In all of the foregoing tests, the grasshoppers were thoroughly coated with the chemical by dipping them in the dust. The excess dust was then immediately removed from their bodies by vigorously jarring the small

cylindrical screen cages as soon as the 'hoppers could be transferred to them. In the subsequent studies, a dusting atomizer was used and both 'hoppers and plants were dusted in large bell jars. Reasonably accurate dosages were obtained by weighing the amount of dust collected on measured squares of paper placed on the floors of the bell jars. In the dusting experiments, 50 grasshoppers, 10 groups of 5 each, were used in each test. As the first two series of experiments showed that the males were more easily killed, only adult females were employed in the dusting experiments.

The data presented in table 3 show that 3,5-dinitro-o-cresol, 2,4-dinitro-o-cyclohexylphenol and 25 per cent sodium arsenite[3] dusts act as both contact insecticides and as stomach poisons. Ten per cent 3,5-dinitro-o-cresol dust applied at the rate of 10 pounds per acre killed all of the 'hoppers in 96 hours and as the rate of application was increased the time required to kill was reduced. When applied as a contact dust (table 3, series A) 5 and 10 per cent 3,5-dinitro-o-cresol dusts gave quicker kills and more effective control than a 25 per cent sodium arsenite dust mixture. When used as stomach poisons (series B) or as both stomach poisons and contact insecticides (series C) the relative toxicity of the two compounds remained about the same.

No large-scale field tests were possible but crude fence row trials, for which no method of measuring the amount of dust used or accurately

TABLE 2. *Average time between treatment and death of Melanoplus bivittatus adults when thoroughly coated with dust mixtures containing various percentages of a chemical compound in diatomaceous earth*

| Compound | Concentration (per cent) | Time to kill (minutes) | | | |
|---|---|---|---|---|---|
| | | Male | | Female | |
| | | Range | Mean* | Range | Mean* |
| 3,5-Dinitro-o-cresol | 100 | 8 - 20 | 14 | 14 - 29 | 21 |
| | 25 | 14 - 25 | 19 | 16 - 58 | 32 |
| | 10 | 14 - 36 | 22 | 31 - 90 | 43 |
| | 5 | 14 - 46 | 23 | 33 - 89 | 45 |
| | 2 | 29 - 51 | 35 | 35 - 127 | 66 |
| | 1 | 45 - 130 | 65 | 65 - 396 | 171 |
| 2,4-Dinitro-o-cyclohexylphenol | 100 | 22 - 42 | 33 | 52 - 97 | 71 |
| | 25 | 71 - 144 | 83 | 156 - 424 | 252 |
| | 10 | 111 - 322 | 150 | 333 - 720 | 544 |
| | 2 | 100 - 675 | 508 | 420 - 1080 | 751 |
| 2,4-Dinitrophenol | 100 | 8 - 19 | 14 | 17 - 34 | 22 |
| | 2 | 113 - 362 | 184 | 116 - 364 | 340 |
| Sodium arsenite | 100 | 14 - 43 | 32 | 18 - 54 | 35 |
| | 25 | 151 - 401 | 240 | 220 - 407 | 330 |

*Each mean obtained from 25 grasshoppers treated and observed individually.

[3] Sodium arsenite dust kindly provided through the courtesy of Dr. Claude Wakeland of the Bureau of Entomology and Plant Quarantine.

TABLE 3. *Percentages of Melanoplus bivittatus adult females dead 12, 24, 48 and 96 hours after treatment; when treated as shown with insecticide dusts of various concentrations*

| Treatment | | | Hours after Treatment | | | |
|---|---|---|---|---|---|---|
| Insecticide | Concentration (percentage) | Pounds per acre | 12 | 24 | 48 | 96 |
| **Series A** | | | | | | |
| 3,5-Dinitro-o-cresol | 10 | 40 | 100* | | | |
| | 10 | 25 | 100** | | | |
| | 10 | 20 | 100 | | | |
| | 10 | 15 | 86 | 100 | | |
| | 10 | 10 | 78 | 92 | 95 | 100 |
| | 10 | 7 | 72 | 82 | 88 | 92 |
| | 5 | 40 | 100 | | | |
| | 5 | 25 | 76 | 100 | | |
| | 5 | 20 | 26 | 60 | 82 | 84 |
| | 2 | 40 | 40 | 80 | 100 | |
| | 2 | 20 | 26 | 56 | 60 | 62 |
| Sodium arsenite | 25 | 15 | 16 | 36 | 60 | 70 |
| | 25 | 10 | 4 | 8 | 26 | 36 |
| **Series B** | | | | | | |
| 3,5-Dinitro-o-cresol | 10 | 10 | 80 | 94 | 100 | |
| 2,4-Dinitro-o-cyclohexlyphenol | 2 | 20 | 40 | 60 | 94 | 100 |
| Sodium arsenite | 25 | 10 | 6 | 34 | 86 | 100 |
| **Series C** | | | | | | |
| 3,5-Dinitro-o-cresol | 10 | 10 | 86 | 100 | | |
| | 10 | 5 | 18 | 46 | 58 | 82 |
| | 2 | 10 | 24 | 42 | 60 | 72 |
| Sodium arsenite | 25 | 10 | 8 | 38 | 88 | 100 |

Series A. Grasshoppers dusted and placed on fresh, unpoisoned food.
Series B. Soybean plants dusted and undusted grasshoppers caged on the plants.
Series C. Grasshoppers and soybean plants dusted together.
*100 per cent dead in 2 hrs.
**100 per cent dead in 4 hrs.
Fifty adult females (10 lots of 5 each) used for each dust concentration.

evaluating results was available, seemed to be in general agreement with the results obtained in the laboratory.

Rough field tests with adult Mormon crickets (*Anabrus simplex* Hald.) confined on wheat stubble by metal barriers showed definitely

that crickets were considerably harder to kill than grasshoppers, but applications of 7 or 8 pounds per acre of 15 per cent 3,5-dinitro-o-cresol dust killed 30 to 40 per cent of the crickets in three hours. In two additional tests, where 20 to 25 pounds of dust were used per acre, 86 and 99 per cent, respectively, of the crickets were killed in three hours.

Young crickets, however, seem to be very easily killed by the dinitro-cresol. First instar Mormon crickets dusted under bell jars in the laboratory were consistently killed in one hour by 3,5-dinitro-o-cresol dusts when applied at the rate of 4 pounds per acre of 5 per cent dust and 3 pounds per acre of 10 per cent dust. Even lower concentrations gave rather high mortalities but the time required was lengthened. First instar grasshoppers, *M. bivittatus*, died even more quickly than the young crickets.

Various concentrations of 3,5-dinitro-o-cresol dusts were tried also on field crickets, weevils, ground beetles, tenebrionids, ants, and several species of Hemiptera and all seemed to be susceptible to the poison. In general, the concentration and quantity of dust required to kill seemed to be correlated with the size of the insect.

In the case of the chinch bug, *Blissus leucopterous* Say, over-wintered adults were consistently 100 per cent killed in 1 hour by dust mixtures applied at the rate of 2 pounds per acre of 10 per cent dust, 3 pounds per acre of 5 per cent dust, or 10 pounds per acre of 2 per cent dust. It must be kept in mind, however, that all dosages were applied to a smooth surface under laboratory conditions and that in all probability considerably higher dosages would be required in the field. The limited supply of the chemicals did not permit field test, except on a very limited basis.

## DISCUSSION

The experimental data in Iowa suggest the possibility of obtaining good kills of grasshoppers in a comparatively short time with a 10 per cent 3,5-dinitro-o-cresol dust applied at the rate of 10 to 15 pounds per acre.

Although the practical use of dinitro-o-cresol as a dust for grasshopper control remains to be established by further study, the results reported in this paper are not inconsistent with data presented by numerous European workers, mostly in Germany, who, in the last few years, have reported varying degrees of success attending the use of dusts containing dinitro-o-cresol for the control of aphids, geometrids, nunmoth, and May beetles. Experimental work for the control of locusts and grasshoppers has been conducted also in Argentina and Africa.

Although 3,5-dinitro-o-cresol is quite insoluble, it reportedly burns considerably some types of foliage. Marcus (1937) reported spruce, larch, beech and birch moderately scorched but meadow grass uninjured. Thiem (1938) says, "Owing to injury to leaves dusting and spraying is not recommended in orchards." Schwerdtfeger (1939) observed, "Dinitro-cresol not only destroys all adults (cockchafers) with which it comes in contact, but as it also kills the young foliage the surviving adults die."

Other workers present conflicting views on the degree of burning produced possibly because of differences in the tolerance of the plants and conditions under which they worked. The sodium salt of 3,5-dinitro-o-cresol which is water soluble is used as a selective weed killer. Westgate and Raynor (1940) report success in controlling mustard, wild lettuce and other broad-leaved weeds without damage to small grains, corn, onions, alfalfa and flax. The same authors point out that the applications recommended (which are higher than would be used in insect control) have no injurious action upon the soil.

Until much more information on the plant tolerance of these chemicals becomes available, their possible usefulness may be restricted largely to the treatment of mass populations of gregarious grasshoppers and other inects where plant-burning would be of secondary consideration. At the same time, if plant tolerance will permit its use, the susceptibility of the chinch bug and many other field crop insects to this material would indicate a possible wide use for 3,5-dinitro-o-cresol on small grain or corn and even in temporary barrier construction.

The dinitro-cresol and phenol compounds are known to be powerful reducing agents and to induce increased rates of metabolism in mammals but there seems to be no agreement in the literature on the probable effect of small concentrations of these materials on man and other vertebrate animals. For the present, they should be used in field experiments only with masks and protective clothing.

Although 3,5-dinitro-o-cresol was the most toxic compound included in this study, dinitrophenol, 2,4-dinitro-6-cyclohexylphenol and the salts of these compounds also need further study because they produced a high mortality but acted more slowly than the former compound.

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# SOME REACTIONS OF GRASSHOPPERS TO CASTOR BEAN PLANTS[1]

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Received May 17, 1940

The wide publicity given castor bean plants, *Ricinus communis* L., as a means of grasshopper control gave rise to the laboratory experiments described below. From articles in popular magazines and newspapers it became evident that many inquiries would be received in regard to the alleged poisonous and protective powers of castor beans applicable to grasshoppers.

In 1931 the Japanese beetle was reported to have been strongly attracted to castor bean plants and killed by feeding on the leaves. Metzger (1933) stated that in cage tests the Japanese beetle fed on castor bean foliage to a limited extent only and that the plant was practically nontoxic. Tests showed that this plant was of little or no value as a trap plant for the beetle under usual field conditions. Myers (1939) summarized the insects attacking the castor bean in the United States and foreign countries. There are very few records in the literature of damage to castor bean plants by grasshoppers and only a few records of insects being poisoned by feeding on the plant.

Inasmuch as the literature has been well reviewed in a recent article by Smith (1939), an extensive review is omitted. Smith stated that grasshoppers lived fairly well on an exclusive diet of castor bean foliage and petioles but they did not relish any part of the castor bean plants for food. Since grasshoppers died in the castor bean test cages at a rate intermediate between the rate on alfalfa and that under starvation conditions, no evidence of poisoning could be observed.

## MATERIALS AND PROCEDURE

An effort was made to obtain seed of a number of varieties of the castor bean, *Ricinus communis* L. Seven varieties were obtained in the United States and an eighth secured from Europe as follows: *R. cambodgensis, zanzibariensis, scarlet queen, africanus, palma christi, borbonensis, sanguineus,* and *laciniatus* (imported). Numerous varieties were found in packages of assorted seeds, and samples of mixed seeds received contained two to five varieties each.

All nymphal stages and adults of the differential grasshopper, *Melanoplus differentialis* (Thomas), were used in the experiments. Some

[1] Journal Paper No. J-749 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 649.

[2] The writer wishes to acknowledge the assistance of Dr. C. J. Drake, under whose direction the work was conducted, also that of Dr. C. H. Richardson and Dr. G. C. Decker in checking the experiments at frequent intervals.

*Melanoplus mexicanus* (Saussure) and *Melanoplus bivittatus* (Say) also were used but the tests were less complete.

The experimental work was conducted in a greenhouse in breeding cages 1 x 1 x 2 feet with soil filled bottoms. Oats, corn, wheat, barley, and castor beans were grown in 5-inch pots. Three series of tests were made as follows, using all available varieties of castor beans in series (1) and only the eight varieties named in the foregoing in series (2) and series (3):

1. Grasshoppers were caged with a pot of cereal plant and a castor bean plant, thus allowing the 'hoppers to feed either on a crop or bean plant.

2. Adults, half-grown nymphs, and first-instar nymphs were placed in cages containing only a single variety of castor bean. For the first-instar 'hoppers, leaf discs of the castor bean were presented to the insects in glass dishes provided with moist blotting paper to prevent desiccation.

3. Newly emerged adult differential grasshoppers reared on corn and oats were transferred to screened cages (18 x 40 x 18 inches) containing eight bean plants—one of each variety. The initial population consisted of 10 males and 10 females per cage. The positions of the plants were changed at intervals within cages and varied between cages.

## EXPERIMENTAL DATA AND RESULTS

Grasshoppers in series (1), which were given an opportunity to feed on both crop plants and castor bean plants, readily ate corn, wheat, oats, and barley in preference to any of the varieties of castor bean. Figure 1 shows the results of selective feeding when differential grasshoppers were offered oat plants and a castor bean plant in the same cage. In some cages as many as four pots of oats or other crop were consumed each day, whereas in only two cases, because of stem injury, was it necessary to replace the castor bean plant during the entire course of the experiments. The grasshoppers were not repelled by the castor beans and often rested on the leaves and stems but seldom ate any portion of the plant.

In tests of series (2), the author was unable to rear young grasshoppers on a diet of castor bean leaves. These first instar nymphs provided with castor bean food lived an average of 4.6 days as compared with 3.0 days for 'hoppers of the same age in check cages without food. Some nymphs refused entirely to eat the bean leaves, whereas others ate small amounts and a few lived to the second instar. They became quite inactive, appearing weak and unhealthy several days before death occurred.

Other *M. differentialis* nymphs were reared to the third and fourth instar stage on corn and oats before being fed exclusively on growing castor bean plants. Among these 'hoppers so limited in a diet of castor bean plants, the mortality was high. Few lived to become adults and then the adult life was short. After being restricted to castor beans, none lived beyond 30 days, whereas in the check cages, an average of 80 per cent were reared to adults, and most of them reproduced. There was no appar-

ent difference in the nutritional value of the eight varieties of castor beans as observed in these tests.

In tests of series (3), in which adult differential grasshoppers had access to the eight varieties of castor bean and no other food, some of the insects died during the first week after being placed on castor beans; half of the population was dead at the end of 29 days but a few individuals lived as long as 60 days. Throughout the tests, these adults were sluggish, quite inactive, and not easily disturbed. Death usually occurred after a day or more of feeble activity suggestive of slow-starvation diet. Feeding was light and in most cases very little of each plant was consumed. Adult differential grasshoppers showed a very slight preference for the *africanus* variety of castor bean as indicated by the number of plants destroyed. In the three cages used, the following varieties were replaced either because of stem injury or defoliation: *africanus*, 7 replacements; *palma christi*, 2 replacements; *cambodgensis* and *sanguineus*, 1 replacement of each; other varieties, no replacements.

## SUMMARY AND CONCLUSIONS

In the tests conducted there was no evidence that grasshoppers were attracted to castor bean plants and common crop plants were selected in preference to all the tested varieties. There was no evidence also of a repellent effect of castor beans since grasshoppers used in these tests often rested on the plants and similarly under field conditions, grasshoppers have been observed to spend the night resting on castor bean plants and then move in the morning to other species of plants to feed. Because grasshoppers were able to survive longer on an exclusive diet of castor bean plants than with no food, it is concluded the poisonous principle of the castor oil plant had very little if any effect on them.

During the present grasshopper outbreak in the United States, considerable attention has been given to the castor bean plant as an alleged killing agent for grasshoppers. However, from the results of the above experiments, castor beans cannot be regarded as having any direct value in grasshopper control or as a measure of preventing grasshopper damage to crops.

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## PLATE I

Fig. 1.—(a). Cage of 100 *M. differentialis* nymphs and adults with two fresh pots of oat plants and one castor bean plant.

(b). Same cage after six hours feeding to show comparative feeding on oats and castor bean plant. Note grasshoppers resting on castor bean plant.

PLATE I

(a). Cage of 100 *M. differentialis* nymphs and adults with two fresh pots of oat plants and one castor bean plant.

(b). Same cage after six hours feeding to show comparative feeding on oats and castor bean plant. Note grasshoppers resting on castor bean plant.

# THE DISSIMILATION OF LEVULOSE BY HETEROFERMENTATIVE LACTIC ACID BACTERIA

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The heterofermentative lactic acid bacteria are unique in their ability to form mannitol from levulose. This characteristic is of interest since other hexoses, differing only slightly from levulose, are not reduced in a similar manner; otherwise, the products of the dissimilation of glucose and levulose are similar, the greatest differences being in the quantitative relationships. Considering this behavior, the question of the similarity of the kinetics of the dissimilative processes of the two sugars, is brought up.

Mazé and Perriér (1903) attempted to explain the differences between the dissimilation of glucose and of levulose through a quantitative study of the fermentation. The dissimilation of levulose was considered to be in part identical with that of glucose; that is, in the formation of lactic acid, ethyl alcohol and carbon dioxide. (Only traces of acetic acid were obtained from glucose by these authors.) Otherwise, the fermentation consisted of the reduction of levulose to mannitol with the simultaneous oxidation of ethyl alcohol to acetic acid. The alcohol was thus an intermediary in the levulose fermentation instead of a final product as in the glucose fermentation. By this method, the lower yield of ethyl alcohol and higher yield of acetic acid from levulose were explained.

Smit (1913) by a very careful quantitative study of the products of levulose fermentation, confirmed the findings of early workers as to the low yields of glycerol and ethyl alcohol.

Peterson and Fred (1920) obtained yields of mannitol accounting for as much as 70 per cent of the levulose fermented. The following schema for the fermentation of levulose was proposed:

$$\underset{\text{levulose}}{C_6H_{12}O_6} + H_2O \rightarrow \underset{\substack{\text{acetic}\\\text{acid}}}{C_2H_4O_2} + \underset{\substack{\text{malic}\\\text{acid}}}{C_4H_6O_5} + 2H_2$$

$$\underset{\substack{\text{malic}\\\text{acid}}}{C_4H_6O_5} \rightarrow \underset{\substack{\text{lactic}\\\text{acid}}}{C_3H_6O_3} + CO_2$$

$$2\,\underset{\text{levulose}}{C_6H_{12}O_6} + 2\,H_2 \rightarrow 2\,\underset{\text{mannitol}}{C_6H_{14}O_6}$$

This schema requires the formation of acetic acid, lactic acid and carbon dioxide in equimolar quantities, equal to one-half the number of mole-

cules of mannitol formed. These requirements are not completely satisfied by the quantitative data of Mazé and Perriér (1903), Pederson (1929), Gayon and Dubourg (1901) and Charleton, Nelson and Werkman (1934).

Kluyver's (1935) view of the dissimilation of levulose by heterofermentative lactic acid bacteria is similar, in part, to that of Mazé and Perriér (1903). In Kluyver's scheme intermediary acetaldehyde instead of ethyl alcohol is oxidized to acetic acid.

Bolcato (1936) suggested that the quantity of lactic acid formed was independent of the quantitative relationships of the other products. The acidity produced had a marked effect on the quantity of other products. The following schema of dissimilation was formulated:

$$3 \text{ levulose} \rightarrow 2 \text{ mannitol} + 2 \text{ acetic acid} + 2\,CO_2$$
$$\text{levulose} \rightarrow 2 \text{ lactic acid}$$

The levulose competes with intermediary acetaldehyde for hydrogen from the breakdown of intermediary pyruvic acid.

These workers seem to agree on the formation of mannitol by a reduction of levulose with activated hydrogen which results from the breakdown of another molecule of levulose. There is little uniformity in thought as to the exact reactions taking place to bring about the formation of this activated hydrogen.

A study by Nelson and Werkman (1936) in which hydrogen acceptors were added to glucose fermentations, suggests the possibility of levulose acting as a hydrogen acceptor in a manner similar to that of acetylmethylcarbinol. When acetylmethylcarbinol was added to glucose fermentations, the yields of ethyl alcohol and glycerol were decreased and the yield of acetic acid increased. The acceptor was reduced to 2, 3-butylene glycol. The differences between the glucose and levulose fermentations are similar to the differences between the normal glucose fermentation and the glucose fermentation to which acceptors had been added.

The present investigation was undertaken to study the mechanism of levulose dissimilation and to compare it with the dissimilation of glucose to which hydrogen acceptors had been added.

## METHODS

Two heterofermentative lactic acid bacteria, *Lactobacillus lycopersici* and *Leuconostoc dextranicus* were used in the investigation.

Three series of fermentations were carried out with each organism. The first series comprised fermentations of levulose as the sole substrate, the second series employed glucose alone, and in the third series, acetylmethylcarbinol was added to the glucose fermentations.

The medium consisted of hexose, 2.0 per cent; yeast extract (Difco) 0.3 per cent; peptone 0.5 per cent; $KH_2PO_4$ 0.6 per cent and $K_2HPO_4$ 0.6 per cent; 0.5 per cent acetylmethylcarbinol was added to the medium of the third series. Solutions of (a) hexose, (b) yeast extract and peptone and (c) phosphates were sterilized 20 minutes at 20 pounds. Acetylmethyl-

carbinol was dissolved in water and sterilized by Seitz filtration. The various solutions were combined at the time of inoculation.

Inoculation was made with 50 ml. of a 3-day culture of the organisms grown on medium of the same composition to be inoculated.

The glucose and levulose fermentations were complete after 21 days' incubation at 30° C. The fermentations of glucose to which acetylmethylcarbinol had been added, were complete in about 12 days.

Complete anaerobiosis was maintained by continuously bubbling oxygen-free nitrogen through the fermenting medium.

## METHODS OF ANALYSIS

The carbon dioxide formed during fermentation was carried into Bowen potash bulbs by a stream of oxygen-free nitrogen, bubbled continuously through the medium, and determined gravimetrically.

The volatile acids were determined by the method of Osburn, Wood and Werkman (1933) and the lactic acid by the method of Friedemann, Cotonio and Shaffer (1927).

The alcohol was oxidized to acetic acid and determined by the method of Stahly, Osburn and Werkman (1934).

Glycerol was determined by the method of Wagenaar (1911) after extracting acetone. Mannitol was extracted with alcohol and determined by the method of Smit (1914).

The hexoses were determined by the Munson and Walker (1906) method.

Acetylmethylcarbinol and 2,3-butylene glycol were determined by the methods of van Niel (1927) and Brockmann and Werkman (1933) respectively as modified by Stahly and Werkman (1936). Corrections for the effect of acetylmethylcarbinol on the determination of glucose, ethyl alcohol and 2, 3-butylene glycol were made.

Purity of the cultures was determined by microscopic and cultural examination at the time of inoculation and just before analysis.

## EXPERIMENTAL

The products of fermentation of glucose and levulose, typical of the two organisms used, are shown in table 1, calculated as m.Mol. per 100 m.Mol. of hexose. There is a marked quantitative difference between the products of the two hexoses. A large quantity of mannitol is formed from levulose but none from glucose. The products of *Leuconostoc dextranicus* and *Lactobacillus lycopersici* are qualitatively alike but differ quantitatively. The two organisms could not be distinguished by their products of dissimilation of glucose, but *Leuconostoc dextranicus* produces less mannitol and more ethyl alcohol from levulose than *Lactobacillus lycopersici* under the conditions of these experiments.

Nelson and Werkman (1936) showed that the addition of suitable hydrogen acceptors to glucose fermentations, resulted in a decrease in the yields of ethyl alcohol and glycerol and a simultaneous increase in

TABLE 1. *Dissimilation of glucose and levulose by heterofermenative lactia acid bacteria*

| | *Leuconostoc dextranicus* | | *Lactobacillus lycopersici* | |
|---|---|---|---|---|
| | Glucose m.Mol. | Levulose m.Mol. | Glucose m.Mol. | Levulose m.Mol. |
| Hexose fermented ..... | 100 | 100 | 100 | 100 |
| Ethyl alcohol ..... | 81.2 | 51.2 | 74.1 | 0.8 |
| Acetic acid ..... | 10.8 | 34.9 | 15.3 | 40.3 |
| Carbon dioxide ..... | 86.5 | 77.5 | 81.0 | 44.7 |
| Lactic acid ..... | 83.5 | 53.4 | 83.1 | 33.1 |
| Glycerol ..... | 24.0 | 2.1 | 32.6 | 3.8 |
| Mannitol ..... | ..... | 29.8 | ..... | 62.3 |
| Carbon recovery, percentage .. | 98.9 | 99.1 | 101.1 | 101.9 |

acetic acid. If it be assumed that part of the levulose acts as a hydrogen acceptor, and the remainder follows the same course of dissimilation as glucose, similar relationships will occur as when hydrogen acceptors are added to glucose fermentations.

To show these relationships the quantity of mannitol formed was assumed to equal the quantity of levulose acting as a hydrogen acceptor. This value was subtracted from the total levulose fermented, giving the quantity of levulose following the same course of dissimilation as glucose. The products were then calculated in terms of 100 m.Mol. of the unreduced levulose.

Typical fermentations of levulose calculated on this basis are compared in tables 2 and 3 with glucose fermentations to which acetylmethylcarbinol has been added. The reduction products of the hydrogen acceptors are now shown in these tables.

There was much less alcohol and glycerol formed from levulose by

TABLE 2. *Comparison of levulose and acetylmethylcarbinol as hydrogen acceptors in fermentations by L. lycopersici*

| Product | Glucose | Glucose + acetylmethylcarbinol | Levulose |
|---|---|---|---|
| | m.Mol. | m.Mol. | m.Mol. |
| Lactic acid ..... | 83.1 | 61.7 | 84.8 |
| Acetic acid ..... | 15.3 | 52.9 | 106.7 |
| Ethyl alcohol ..... | 74.1 | 54.8 | 2.1 |
| Carbon dioxide ..... | 81.0 | 100.1 | 118.2 |
| Glycerol ..... | 32.6 | 25.3 | trace |
| Carbon recovery, percentage ..... | 101.1 | 95.3 | 98.3 |

TABLE 3. *Comparison of levulose and acetylmethylcarbinol as hydrogen acceptors in fermentations by L. dextranicus*

| Product | Glucose | Glucose + acetyl-methylcarbinol | Levulose |
|---|---|---|---|
| | m.Mol. | m.Mol. | m.Mol. |
| Lactic acid | 83.5 | 78.0 | 76.0 |
| Acetic acid | 10.8 | 38.9 | 50.4 |
| Carbon dioxide | 86.5 | 103.1 | 111.8 |
| Ethyl alcohol | 81.2 | 67.4 | 74.4 |
| Glycerol | 24.0 | 18.5 | trace |
| Carbon recovery, percentage | 98.8 | 100.8 | 98.2 |

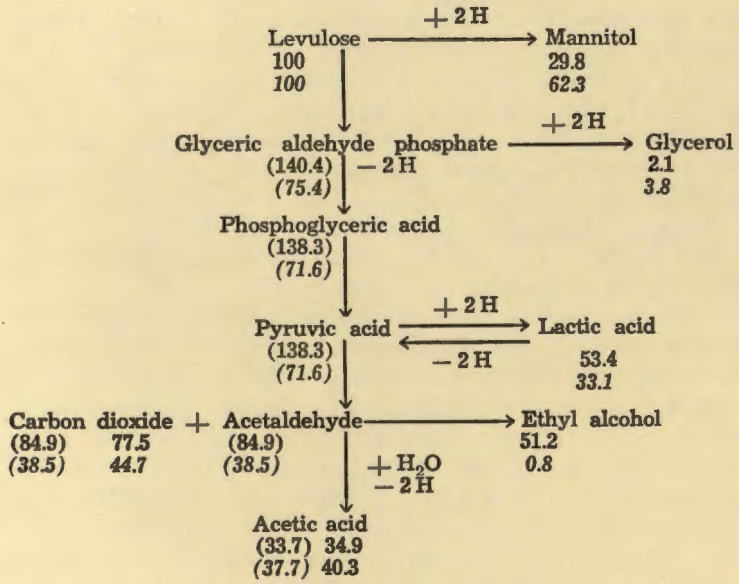
both organisms than from glucose. The quantity of acetic acid on the other hand was much greater in the levulose fermentation. These differences were more pronounced in the fermentations by *Lactobacillus lycopersici* than those by *Leuconostoc dextranicus*. The latter produced an appreciable quantity of ethyl alcohol from levulose. Whether this is characteristic of Leuconostoc has not been determined.

The relationships between the levulose fermentation and the glucose fermentation are very similar to those between the glucose fermentation plus acetylmethylcarbinol and the normal glucose fermentation. Assuming levulose to be an acceptor, added to a hexose fermentation, both acceptors suppressed the formation of ethyl alcohol and increased the formation of acetic acid. In the case of *L. lycopersici* the formation of ethyl alcohol was entirely prevented by levulose. The acetic acid was increased in the fermentations by *L. lycopersici* from 15 m.Mol. (glucose) to 52.9 m.Mol. with the acetylmethylcarbinol and 106.7 m.Mol. with the levulose. The ethyl alcohol decreased from 74.1 m.Mol. in the glucose medium to 54.8 m.Mol. with the acetylmethylcarbinol and 2.1 with the levulose. The same relationships exist in the fermentations by *Leuconostoc dextranicus*. Ethyl alcohol decreased from 81.2 m.Mol. (glucose) to 67.4 m.Mol. with acetylmethylcarbinol and 74.4 m.Mol. with levulose. Yields of acetic acid were 10.8 m.Mol. from glucose, 38.9 m.Mol. with acetylmethylcarbinol and 50.4 m.Mol. with levulose. These relatonships indicate that levulose and acetylmethylcarbinol are playing similar roles in the dissimilation of carbohydrates. Apparently the same intermediary is oxidized in the levulose as in the "glucose plus acetylmethylcarbinol" fermentation to bring about the increase in acetic acid at the expense of ethyl alcohol. It is very likely that the same intermediate products are formed in the dissimilation of levulose as are formed in the dissimilation of glucose. These intermediates have not been definitely determined, but a schema of dissimilation is proposed in figure 1. The data from table 1 are applied to the schema. It will be noted that the values found and calculated are in good agreement.

SUMMARY

A comparison of the dissimilation of levulose with that of glucose plus hydrogen acceptors by heterofermentative lactic acid bacteria (*Lactobacillus lycopersici* and *Leuconostoc dextranicus*) indicated that the two types are similar. This relationship suggests that in the fermentation of levulose the ketose is functioning simply as an acceptor to compete with normally formed intermediary acceptors and that the mechanism of dissimilation of the levulose not acting as an acceptor is quite similar to that of glucose. In both cases, the production of ethyl alcohol and glycerol was reduced by acceptors; acetylmethylcarbinol in the case of glucose to form 2, 3-butylene glycol and levulose, acting as its own acceptor, to form mannitol. On the other hand, the yield of acetic acid was increased according to expectations.

The path of dissimilation of the oxidized levulose is apparently the same as that of glucose under normal conditions.




Values in italics are data for *Leuconostoc dextranicus;* values not italicized are for *Lactobacillus lycopersici.* The values in parentheses are calculated; others are experimental.

Fig. 1. Schema of the dissimilation of levulose by *Lactobacillus lycopersici* and *Leuconostoc dextranicus.*

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# NUTRITIVE REQUIREMENTS OF THE HETEROFERMENTATIVE LACTIC ACID BACTERIA[1]

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Received May 17, 1940

It is of deep concern that the principles of cellular nutrition be made clear, inasmuch as such knowledge is essential to an understanding of the mechanism of cell metabolism. There has been considerable renewed interest recently in the determination of the nutritive requirements of bacteria. The excellent reviews by Knight (1936), Burrows (1936) and Koser and Saunders (1938) summarize all but the latest literature in this field. Briefly the status of the problem with respect to the lactic acid bacteria may be summarized as follows: Orla-Jensen, Otte and Snog-Kjaer (1936) showed that riboflavin and one or more other "activators" are necessary for growth of certain lactic acid bacteria. They concluded from questionable evidence that one of these substances is pantothenic acid. Subsequently Snell, Strong and Peterson (1938, 1939) substantiated this conclusion by using purified preparations of pantothenic acid and showed that nicotinic acid together with pantothenic acid was essential for growth of two species in a hydrolyzed casein medium but additional factors were necessary for other species. Wood, Andersen and Werkman (1937), using a basal medium containing glucose, hydrolyzed casein, inorganic salts, vitamin $B_1$ and ether extract of yeast extract, confirmed the conclusion of Orla-Jensen et al. that riboflavin is required by the lactic acid bacteria. The activity of the ether soluble factor had previously been shown by Wood, Tatum and Peterson (1937) for the propionic acid bacteria, and by Snell, Tatum and Peterson (1937) for the lactic acid bacteria. Möller (1938) found, that in addition to the ether soluble acid and alkali labile factor of Snell et al., crystalline vitamin $B_6$ is essential. The same author (1939) established biotin as an essential factor and that in addition to vitamin $B_6$, nicotinic acid, thiamin, riboflavin, β-alanine and unknown factors F (probably pantothenic acid), G and H are influential.

The amino acid requirements of the lactic acid bacteria have not been investigated extensively. Orla-Jensen et al. (1936) studied a large number of species. However, from the standpoint of determination of essential amino acids, the work is not conclusive, since the basal medium contained whey and it is doubtful whether the whey was free from traces of amino acids.

The bacteria used in this investigation were the heterofermentative lactic acid bacteria: *Lactobacillus mannitopoeus* L2), *L. buchneri* (L4) and *L. lycopersici* (L5). Under the conditions of our experiments in a

[1] Journal Paper No. J-690 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 421.

medium containing glucose as a source of carbon these cultures require for optimum growth; inorganic salts, amino acids, ether extract of yeast extract, thiamin and riboflavin. The ether soluble extract is not replaceable by a combination of pantothenic acid, nicotinic acid, vitamin $B_6$, β-alanine, pimelic acid and uracil. Apparently the growth requirements differ from those of the species used by Snell et al. (1938) and Möller (1938), who found factors in addition to ether extract of yeast extract to be essential. According to Orla-Jensen's classification our cultures would be included in *Betabacterium*. Orla-Jensen et al. did not study the amino acid requirements of this genus. In our tests the homofermentative lactic acid bacteria (*L. casei* and *L. delbrückii*) were unable to grow in the amino acid medium, whereas, some cultures of *Streptococcus paracitrovorus* did grow. These streptococci apparently belong to the group studied by Eagles, Okulitch and Kadzielawa (1938).

The purpose of the present investigation was to determine the effectiveness of thiamin, riboflavin and ether extract of yeast extract in stimulating growth in an amino acid medium, and particularly to ascertain which amino acids are influential in promoting growth and acid production.

## EXPERIMENTAL

*Media.* The constituents were used in the following concentrations unless otherwise stated: glucose 1.0 per cent; $(NH_4)_2SO_4$ 0.3 per cent; sodium acetate 0.6 per cent; inorganic salts, $K_2HPO_4$ 0.025 per cent, $KH_2PO_4$ 0.025 per cent, $MgSO_4.7H_2O$ 0.010 per cent, NaCl 0.005 per cent, $FeSO_4.7H_2O$ 0.005 per cent and $MnSO_4.4H_2O$ 0.005 per cent; hydrolyzed casein 0.15 per cent plus tryptophane 0.005 per cent or a mixture of amino acids containing 0.00375 per cent of each amino acid except cystine, which was 0.0005 per cent. The concentrations of amino acids were on the basis of the naturally occurring isomer. Thiamin was used in a concentration of .01 μg per ml.; riboflavin .05 μg per ml.; ether extract of 7.5 mg. of Difco yeast extract per ml.

The hydrolyzed casein was prepared by acid hydrolysis of Glaxocasein A/E at 120°C. for 10 hours. The sulfuric acid was neutralized with $Ba(OH)_2$ and the $BaSo_4$ removed by filtration. The final pH was adjusted with sodium hydroxide solution to 6.8. The amino acids were purchased from the University of Illinois and Eastman Kodak Company and consisted of the following: glycine, dl-alanine, dl-valine, dl-leucine, dl-isoleucine, dl-phenylalanine, l-tyrosine, l-proline, l-hydroxyproline, d-glutamic acid, l-aspartic acid, l-arginine, dl-lysine, l-histidine, dl-threonine, dl-methionine, l-cystine, l-tryptophane, dl-serine. The thiamin (Eastman) and riboflavin (Bordon) were crystalline. The ether extract of yeast extract was prepared as described by Wood, Tatum and Peterson (1937) with the exception that a large part of the succinic acid was removed by holding the extract in ether in the refrigerator for 48 hours and discarding the separated crystals. In the experiments in which replacement of the ether soluble factor by other growth factors was attempted, the medium contained the following micrograms of each substance per ml. of medium:

pantothenic acid[2] 0.2, nicotinic acid 2.0, β-alanine 2.0, vitamin $B_6$ 0.96, pimelic acid 2.0, uracil 6.0. The media were tubed in 5 ml. portions in 13 mm. x 150 mm. tubes and autoclaved at 15 lbs. for 20 minutes.

*Inoculum.* Bacteria for the inoculum were grown in the above described hydrolyzed casein medium and carried through at least three transfers before use. Centrifuged cells from a 4 or 5 day culture were washed once with a volume of water equivalent to that of the original medium and then suspended in an equal volume of water. One drop of this suspension was used per 5 ml. of medium.

*Incubation and measurement of growth.* Incubation was at 30°C. for 5 days under cotton plugs. Growth was measured by titrating the acids formed with 0.05 N alkali using bromothymol blue indicator.

*Stimulating effect of accessory factors.* Table 1 shows the influence of riboflavin, thiamin, ether extract of yeast extract and tryptophane on acid production by the three cultures considered in this investigation. Six different media were tested: (1) the complete medium alone and with the following omissions: (2) riboflavin, (3) thiamin, (4) thiamin and riboflavin, (5) ether extract of yeast extract and (6) tryptophane. The results from four serial transfers are given in ml. of 0.1 N acid per 10 ml. of medium. Consider first the results with culture L4, *Lactobacillus buchneri.* The omission of riboflavin did not retard the growth, from which it may be concluded that the compound is not an essential constituent of the medium and furthermore does not stimulate growth when present. The removal of thiamin decreased the acid production some-

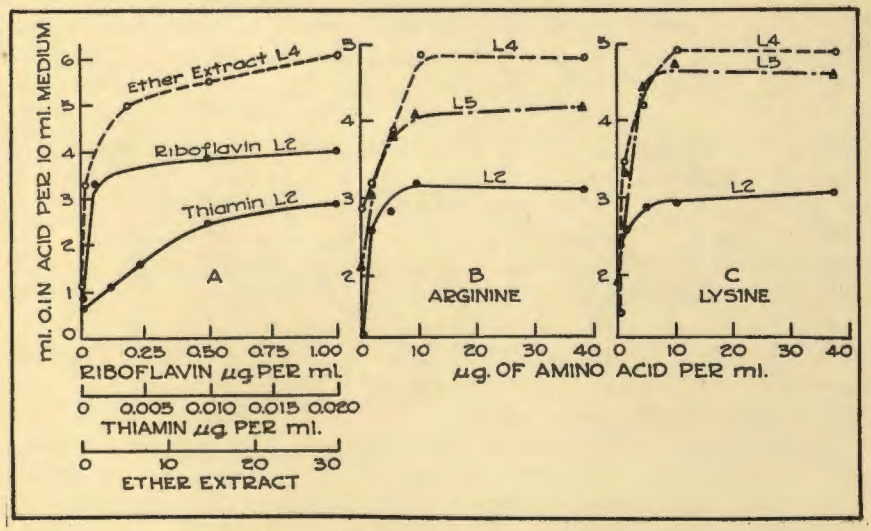


Fig. 1. The effect of variation of the concentration of accessory factors and arginine and lysine on production of acid.

[2] Appreciation is expressed to Dr. R. J. Williams for supplying a preparation of pantothenic acid.

TABLE 1. *Effect of riboflavin, thiamin, ether extract of yeast extract and tryptophane on acid production by the heterofermentative lactic acid bacteria in hydrolyzed casein plus tryptophane medium*

| Culture number | L2 | | | | L4 | | | | L5 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of transfers | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Compound omitted from medium | ml. of 0.1 N acid/10 ml. of medium | | | | | | | | | | | |
| None | 2.3 | 2.5 | 2.3 | | 4.5 | 3.9 | 4.7 | | 2.2 | 2.5 | 2.2 | |
| Riboflavin | 2.2 | 1.9 | 1.9 | 1.9 | 4.7 | 4.8 | 5.2 | 4.0 | 1.7 | 1.8 | 1.7 | 1.9 |
| Thiamin | 1.8 | 1.8 | 1.8 | 1.9 | 3.0 | 3.5 | 3.9 | 3.5 | 1.3 | 1.4 | 1.4 | 1.7 |
| Thiamin and riboflavin | 0.4 | 1.3 | 0.1 | 0.0 | 3.5 | 3.2 | 3.6 | 3.5 | 0.9 | 0.2 | 0.0 | |
| Ether extract of yeast extract | 1.0 | 0.1 | 0.0 | | 1.7 | 0.2 | 0.0 | | 0.9 | 0.2 | 0.0 | |
| Tryptophane | 2.6 | 2.7 | 2.8 | 3.0 | 3.4 | 1.8 | 0.9 | 0.5 | 1.8 | 2.1 | 2.7 | 2.6 |

Note: The values reported in the tables are not always comparable for different experiments, apparently because there is some variation in the activity of the bacteria. The cultures were not very active at the time of this experiment.

what but it was not essential for growth. When ether extract of yeast extract is omitted, growth is no longer obtained on serial transfer. Ether extract of yeast extract is essential for culture L4 *(L. buchneri)* and likewise for cultures L2 *(L. mannitopoeus)* and L5 *(L. lycopersici)*. Both riboflavin and thiamin stimulate growth of cultures L2 and L5 but neither is essential in the presence of the other. When both compounds are omitted, continuous growth is no longer possible. Apparently thiamin and riboflavin can reciprocally replace each other in this case.

Figure 1A shows the effect of varying the concentration of the ether extract, riboflavin and thiamin. Ether extract of yeast extract approaches its full effect when the ether extract from 6.0 mg. of Difco yeast extract is added per ml. of medium. Riboflavin is almost fully effective at 0.05 microgram per ml. and thiamin at 0.01 microgram per ml. The concentration of thiamin was reduced to 0.0025 microgram per ml. in the riboflavin tests and the riboflavin to .01 microgram in the thiamin tests in order to reduce the growth at zero concentration. This procedure produces clear cut results. Also, it is sometimes helpful to use a serial transfer to deplete the carry-over in the inoculum. However, occasionally this practice is unsuccessful, inasmuch as the bacteria may adapt themselves to growth in the absence of thiamin or riboflavin during the transfers (Wood, Andersen and Werkman, 1938; Silverman and Werkman, 1939). It is essential that the inoculum consist of bacteria in a proper physiological state in order for the above influences to be shown. In our experiments this has been accomplished by carrying the cultures in the hydrolyzed casein medium for some time previous to use in the test.

*Amino acid requirements.* The determination of the amino acid requirements of bacteria is complicated somewhat by variation in the physiology of the organism. The cultures tested have shown reasonably constant requirements, probably because the inoculum always has been grown in hydrolyzed casein plus tryptophane medium, hence no demand is placed on the culture to adapt itself to growth in the amino acid deficient medium. A washed suspension of the cells has been used to minimize carry-over in the inoculum. Depletion by serial transfer was not employed in order to reduce the chance of adaptation by the organism.

The nineteen amino acids were split into the six groups shown in table 2. The first group contains the five amino acids which had proved to be essential in preliminary experiments; the second group, the basic amino acids; the third, the dicarboxylic amino acids; the fourth, the ring compounds other than tryptophane; and the fifth and sixth groups comprised the remaining less complex amino acids. A survey of the results in table 2 shows that omission of any one of the six groups caused a reduction in the quantity of acid produced. Apparently each group contains an amino acid which is necessary for optimal growth.

Inasmuch as all of the groups were essential, the effect of omission of each amino acid singly from the mixture of nineteen was determined. The results are shown in table 3. Of the total group of nineteen, only glycine, leucine, isoleucine, proline and hydroxyproline were without

TABLE 2. *Effect of omission of groups of amine acids from the mixture of nineteen*

| Amino acids omitted from the mixture of nineteen | Culture number | | |
|---|---|---|---|
| | L2 | L4 | L5 |
| | ml. of 0.1 N acid/10 ml. of medium | | |
| None | 4.3 | 6.0 | 4.5 |
| Threonine, methionine, tryptophane, cystine, serine | 0.5 | 0.9 | 0.7 |
| Arginine, lysine, histidine | 1.1 | 2.2 | 0.6 |
| Glutamic acid, aspartic acid | 0.7 | 0.9 | 0.9 |
| Phenylalanine, tyrosine, proline, hydroxyproline | 1.5 | 4.2 | 1.4 |
| Glycine, alanine, valine | 0.9 | 1.5 | 0.9 |
| Leucine, isoleucine | 2.7 | 2.7 | 2.6 |

significant effect on all three cultures. The basic amino acids did not give uniform results on all cultures except in the case of arginine. Growth was less vigorous in the absence of arginine. Omission of lysine retarded growth of cultures L4 and L5. Tryptophane apparently is not essential for cultures L2 and L5 but is for culture L4. The influence of removal of tryptophane is shown more clearly in table 1 in which the tryptophane is depleted by serial transfer. There was no growth in the case of culture L4 after the fourth transfer.

The results in table 2 show that when both leucine and isoleucine are omitted growth is poor, yet in table 3 omission of either of these amino acids singly has little effect. Apparently the two amino acids may be used interchangeably. Table 4 confirms this suggestion and shows further that glycine, proline and hydroxyproline may be omitted with no decrease in growth by the three cultures. Fourteen amino acids plus leucine or isoleucine or both give within experimental error the same results as the nineteen amino acids.

The results with the basic amino acids are less conclusive (table 3) than with the others. Therefore the possible combinations of basic amino acids were tested by addition to a mixture of the remaining twelve amino acids that had been found necessary for optimal growth. The results (table 5) show that no one of the three basic amino acids can replace the complete group for any one of the three cultures although lysine is fairly effective for culture L4. Apparently the mixture of all three basic amino acids is better than any other combination but arginine plus lysine can nearly replace the function of the three.

The effect of varying the concentration of the individual amino acids in the presence of a constant concentration of the remaining eighteen (.0037 per cent except cystine) is shown in figure 2. Glycine, isoleucine, proline, hydroxyproline and histidine were omitted in the tests with arginine and lysine (figures 1B and 1C). It is apparent that each amino

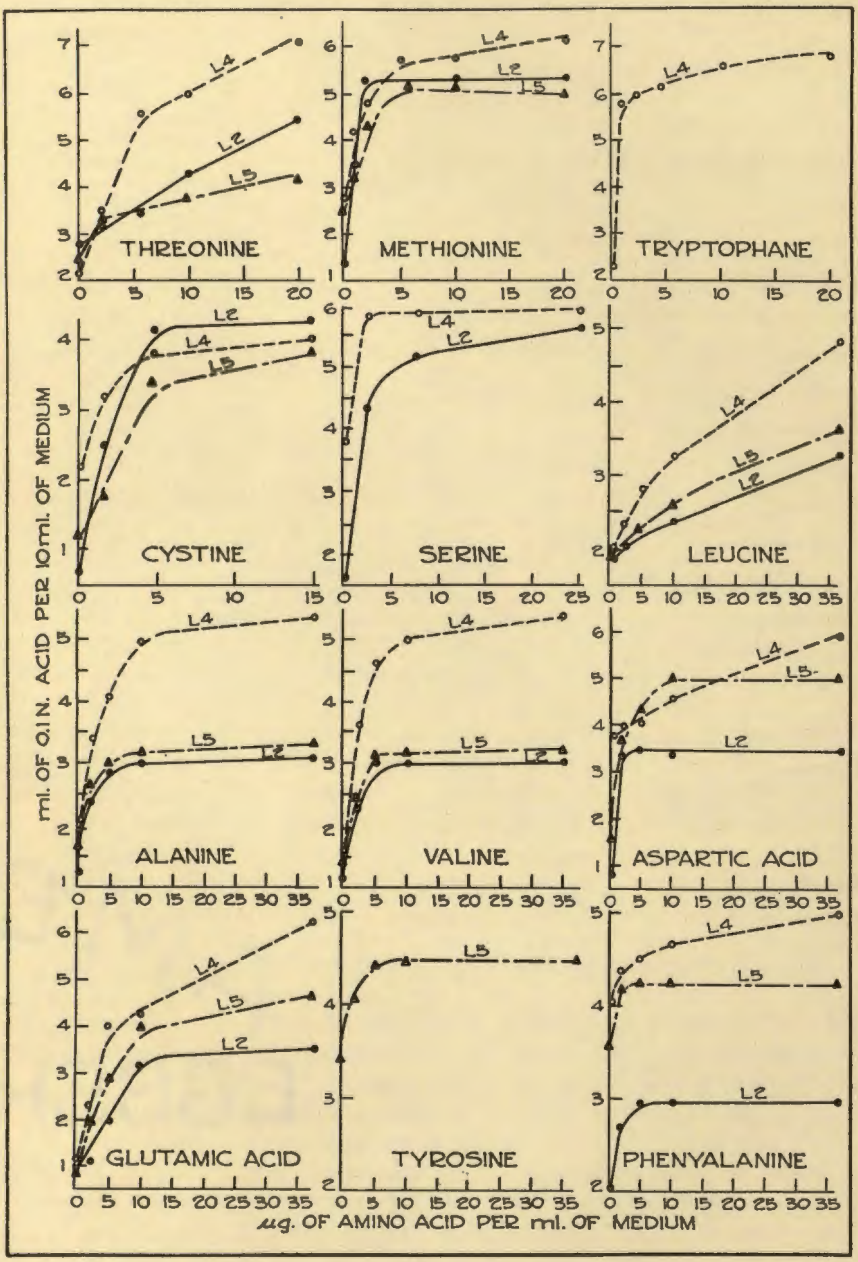


Fig. 2. The effect of variation of the concentration of amino acids on production of acid.

TABLE 3. *Effect of omission of the individual amino acids from the mixture of nineteen*

| Amino acid omitted | Culture number | | |
|---|---|---|---|
| | L2 | L4 | L5 |
| | ml. of 0.1 N acid/10 ml. of medium | | |
| None | 4.4 | 6.1 | 4.5 |
| Serine | 1.5 | 4.2 | 1.7 |
| Tryptophane | 3.9 | 1.6 | 3.9 |
| Methionine | 2.4 | 3.8 | 2.0 |
| Threonine | 1.1 | 0.8 | 1.2 |
| Cystine | 0.7 | 2.1 | 1.6 |
| Arginine | 2.8 | 4.0 | 3.0 |
| Lysine | 4.1 | 2.4 | 3.4 |
| Histidine | 4.1 | 4.4 | 4.4 |
| Glutamic acid | 1.1 | 0.6 | 0.5 |
| Aspartic acid | 0.9 | 2.1 | 0.6 |
| Phenylalanine | 1.3 | 4.35 | 2.8 |
| Tyrosine | 1.7 | 3.9 | 3.0 |
| *Proline | 3.8 | 5.6 | 4.2 |
| *Hydroxyproline | 3.9 | 5.4 | 4.3 |
| *Glycine | 3.9 | 6.7 | 3.8 |
| Alanine | 1.7 | 3.6 | 1.5 |
| Valine | 1.2 | 1.7 | 1.2 |
| *Leucine | 4.1 | 6.0 | 4.3 |
| *Isoleucine | 5.0 | 6.4 | 4.5 |

* Amino acid non-effective in this test for any of the three cultures.

acid reaches its maximal influence on acid production at a low concentration, approximately as follows, the quantities are expressed in micrograms per ml.: threonine 10 to 20, methionine 5, tryptophane 2, cystine 4.5, serine 2.5 to 7.5, leucine 37.5 (in absence of isoleucine), alanine 10, valine 5 to 10, aspartic and glutamic acids 10 for cultures L2 and L5 and 37.5 for L4, tryosine 5, phenylalanine 5 to 10, arginine 10 and lysine 10. A mixture of these fourteen amino acids in the above concentrations was found to support growth almost equivalent to that obtained in the mixture of nineteen amino acids with .0037 per cent of each except cystine. There was considerable variation in the maximal yield of acid, which occurred in spite of the fact that the media were usually identical in the different experiments. These variations were caused largely by change which oc-

TABLE 4. *Effect of addition of leucine and isoleucine to a mixture of fourteen amino acids**

| Amino acid mixture | Culture number | | |
|---|---|---|---|
| | L2 | L4 | L5 |
| | ml. of 0.1 N acid/10 ml. of medium | | |
| 14 amino acids* | 1.9 | 2.4 | 2.0 |
| 14 amino acids* + leucine | 3.5 | 6.5 | 4.0 |
| 14 amino acids* + isoleucine | 4.1 | 6.0 | 4.0 |
| 14 amino acids* + leucine and isoleucine | 3.1 | 5.9 | 3.2 |
| 19 amino acids | 4.2 | 6.2 | 4.1 |

* Serine, tryptophane, methionine, threonine, cystine, alanine, valine, arginine, lysine, histidine, glutamic acid, aspartic acid, phenylalanine and tyrosine.

curred in the activity of the cultures during the two-year duration of the investigation. For this reason the ordinates are plotted to different scales in order to show the activity of the respective amino acids on a comparable basis.

## DISCUSSION

It has been stated in the Introduction that the ether extract of yeast extract is not replaceable by a mixture of known growth factors including among others, vitamin $B_6$, pantothenic acid and nicotinic acid. Growth is

TABLE 5. *Effect of the addition of arginine, lysine and histidine to twelve* amino acids**

| Amino acids added | Culture number | | |
|---|---|---|---|
| | L2 | L4 | L5 |
| | ml. of 0.1 N acid/10 ml. of medium | | |
| Arginine, lysine and histidine | 3.5 | 4.9 | 4.5 |
| Histidine | 1.5 | 1.7 | 1.6 |
| Lysine | 1.8 | 3.1 | 2.5 |
| Arginine | 2.3 | 1.3 | 1.9 |
| Arginine and lysine | 3.2 | 4.3 | 4.1 |
| Arginine and histidine | 2.5 | 1.2 | 2.4 |
| Lysine and histidine | 2.0 | 2.2 | 2.3 |
| None | 1.4 | 0.9 | 1.3 |

* Serine, tryptophane, methionine, threonine, cystine, alanine, valine, glutamic acid, aspartic acid, phenylalanine, tyrosine and leucine.

TABLE 6. *Comparison of the amino acid requirements of various organisms*

| Lactic acid bacteria | Diphtheria bacillus H. Y. strain | Diphtheria bacillus P. W. strain | *Clostridium sporogenes* |
|---|---|---|---|
| Alanine | | | |
| Valine | Valine | Valine | Valine |
| Leucine or isoleucine | | Leucine | Leucine |
| Glutamic acid | Glutamic acid | Glutamic acid | |
| Aspartic acid | | | |
| Cystine | Cystine | Cystine | Cystine |
| Methionine | Methionine | Methionine | Methionine |
| Serine | | | |
| Threonine | | | |
| Phenylalanine | Phenylalanine | | Phenylalanine |
| Tyrosine | | | Tyrosine |
| Tryptophane | Tryptophane | | Tryptophane |
| Arginine | | | Arginine |
| Lysine | | | Histidine |
| | Histidine | | |
| | Glycine | | |

not completely absent, however, in the presence of these factors on the amino acid medium containing thiamin and riboflavin. In fact, cultures L2 and L4 have been carried through as many as twelve transfers in medium completely lacking in the ether extract but in no case has the growth been luxuriant. There is apparently a factor in the ether extract necessary for optimal growth of these bacteria, which is not supplied by the known factors so far tested. It is probable that pantothenic acid and some of the other factors are important for these bacteria.

Orla-Jensen et al. (1936) claim that thiamin and tryptophane are not important in the nutrition of the lactic acid bacteria. The results presented in table 1 show that this does not hold true for all lactic acid bacteria (L4). Furthermore, some lactic acid forming streptococci require tryptophane and thiamin (unpublished data). Snell, Strong and Peterson (1937) have shown tryptophane to be essential for growth of the homofermentative lactic acid bacteria. It is probable that Orla-Jensen et al. did not have a basal medium completely free from thiamin and tryptophane.

The amino acid requirements of the three cultures studied are more complex than found by other workers for different bacteria. Mueller ($1935_1$), ($1935_2$) has studied the requirements of the Ho Yu strain and the Park-Williams strain of diphtheria bacillus and Fildes and Richardson (1935) those of *Clostridium sporogenes*. A comparison of the requirements of these bacteria with those of our cultures is shown in table 6. Alanine, aspartic acid, serine and threonine were not required by either the diphtheria bacillus or *Cl. sporogenes*. The authors have found no previous record of results showing that serine or threonine is required by bacteria. Threonine is decidedly essential for optimal growth of these bacteria. This fact might well be used as the basis of a qualitative and quantitative determination of this amino acid. The extensive list of amino

acids found important for the lactic acid bacteria may be due in part to the method of determining growth. Two factors are involved in our measurement, growth and acid production. Acid production is very closely correlated with growth, as judged by the turbidity of cells. By inspection of the titrations, however, it is apparent that some of these amino acids are not essential for moderate growth. Each of the fourteen amino acids (table 6) has a definitely specific action as shown by the concentration curves (figures 1B, 1C and 2). These amino acids certainly play an important role in the physiology of these bacteria, although perhaps not in the same sense as measured by Mueller or Fildes and Richardson. Adaptation of these bacteria to a simpler amino acid medium or perhaps to a medium containing ammonium salts as the sole source of nitrogen has not been studied extensively. However, preliminary results indicate that the bacteria are rather stable in their amino acid requirements and not as readily subject to quick training to growth on simple nitrogen sources as found by Gladstone (1937) with *Staphylococcus aureus* and Wood, Andersen and Werkman (1938) for propionic acid bacteria.

## SUMMARY

The nutritional requirements of three cultures of heterofermentative lactic acid bacteria have been studied to determine their accessory growth factor and amino acid requirements. Riboflavin or thiamin, and factors occurring in ether extract of yeast extract are necessary for maximal growth of these bacteria in an amino acid medium. Twelve amino acids: alanine, valine, glutamic acid, aspartic acid, cystine, methionine, serine, threonine, phenylalanine, tyrosine, arginine and lysine are essential; that is, omission of any one of these from the medium retards growth and acid production. In addition either leucine or isoleucine must be added, the two apparently are interchangeable. Tryptophane is essential for one culture, L4, the other two cultures can dispense with it.

Our appreciation is expressed to Mr. A. A. Andersen for assistance in some of the early experiments.

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# THE QUANTITY OF PERICARP IN SEVERAL HYBRID AND INBRED STRAINS OF SWEET CORN[1]

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Received June 5, 1940

The changes that occur in the outer layers of the sweet corn kernel, especially the hull, or more correctly the pericarp, are of major interest to the canner and the breeder. Toughness in canned or fresh sweet corn is generally attributed to the toughness of the pericarp. During the processing of corn for canning, the canner can control to some degree the flavor, sweetness and consistency, but no practical methods have been devised for tenderizing the kernel which at the same time do not injure quality. Of course, corn becomes tougher as it passes the prime stage of maturity, but at the prime canning stage (70-72 per cent moisture) practically all strains of sweet corn are tougher than desired.

For determining the degree of tenderness of the pericarp of sweet corn kernels, the puncture tester described by Magoon and Culpepper (1) has been used extensively. A comparison of varieties by this method is not accurate unless all kernels measured are of the same degree of maturity; that is, have the same moisture content. The sweet corn breeder can control toughness to a limited extent as shown by Johnson and Hayes (3) in a study of the inheritance of pericarp tenderness in sweet corn. They crossed a very tender open-pollinated variety and a tough pericarp Crosly inbred. The two parents used in the cross showed a consistent difference of approximately 100 units as measured with a puncture tester, while the $F_1$ cross was intermediate in puncture test value. The same investigators found a daily increase of 20-30 units in puncture test values 18-22 days after pollination. Doxtater (2) also concluded that inbred lines showing low puncture indexes tended to produce crosses having a low puncture index.

The canner and the sweet corn breeder are not directly interested in either the very early or the very late stages of the development of the corn kernel, but an understanding is essential for interpretation of the intermediate stages.

Following fertilization the ovule and its integuments are displaced by the enlarging embryo and endosperm. Concurrent with this is the gradual change of the ovary wall into the pericarp. Ten to 20 days after fertilization, the growth of the entire kernel is very rapid; near the eighteenth day there remains only a very limited peripheral area of nucellus; the integuments as definite layers of tissue have almost disappeared, leaving here and there small masses of disintegrating protoplasm between the

[1] Journal Paper No. J-764. Iowa Agricultural Experiment Station. Project 607.

pericarp and the outer epidermis of the nucellus, and the pericarp has attained its maximum thickness. In the period of 20 to 40 days after fertilization, both the embryo and endosperm continue to enlarge, the epidermis of the endosperm differentiates into aleurone layer, the remainder of the nucellus disappears, except for its outer epidermal wall, and the pericarp is changed to a thin layer. In regard to integuments, it was formerly believed that in corn, like in cereals, the integument tissue persisted to maturity, forming a definite layer; but later work of several investigators (4, 5, 6) indicates that the integuments of the ovule disintegrate and virtually disappear except for scattered non-cellular, non-continuous remnants. The nucellar membrane is presumed to follow in the same manner.

The pericarp, which at the time of fertilization consists of thin-walled parenchyma cells, has increased at the end of 10 days to twice its original thickness. In the crown region of the kernel, the pericarp increases in thickness up to the ninth or twelfth day, and in the basal region to the fifteenth or eighteenth day. About 10 days after fertilization, the middle cells of the pericarp begin to disintegrate in the crown, thereby forming an inner and an outer region or epidermis. Gradually this partial collapse in the middle region extends down the sides of the kernel. After the twentieth day, the outer pericarp increases in size by developing relatively thick cell walls, the cells remaining intact as the kernel expands. In contrast, the cells of the inner pericarp, failing to increase in size, become separated. For a while the cells of the inner epidermis elongate sufficiently to maintain their continuity as a layer of cells, but eventually even these are drawn apart laterally. In the final stage the tangential walls of the outer pericarp continue to thicken, and compression of the entire pericarp occurs, first of the inner layer and then of the entire tissue, to form a tough protective covering. The following problem was undertaken to determine if there were differences in the amount of pericarp between tender and tough strains of sweet corn at the edible or canning stage.

## PROCEDURE

The ear corn was husked and all the silk was picked from the ear. A razor blade or other sharp cutting instrument was used to cut the corn one row at a time just above the tipcap. Slightly more than 1000 grams of kernels were cut off the cob and thoroughly mixed to insure uniform sample. Two moisture samples, consisting of approximately 60 grams each, were weighed out and dried to constant weight in a vacuum oven at 75° C. and approximately 80 mm. pressure.

An 800-gram sample was taken for the pericarp analysis. The sample was first washed with distilled water by rubbing the kernels together lightly in a pan of water. A single layer of kernels was spread on oilcloth and lightly crushed to free the germ from the kernel. The loose germs were then quite readily removed by washing with a rotary motion under a stream of water in an evaporating dish covered with a 4-mesh screen having a metal side 1 to 2 inches deep. About 200 grams of the sample

were used in each washing. Since the value of this procedure depends to a large extent upon securing comparable results, it was necessary to standardize the procedure. However, the standardization was flexible enough to allow for the difference in tenderness and toughness of the samples. The most important part of the procedure was the separation of the endosperm and aleurone from the pericarp. As the endosperm and aleurone were separated by mechanical force, it was necessary to use great care in keeping the pericarp from being ground up at the same time.

The separation of pericarp from endosperm and aleurone took place in a food press which was operated by hand. Instead of grinding all the samples the same length of time, they were ground in the food press until nearly free of endosperm and aleurone. With tough pericarp samples, it was necessary to grind for a longer time as compared to medium or tender pericarp samples. In this separation, factors to be considered are as follows: (a) The amount put in the food press at one time. (b) The length of time of grinding between intermittent washings. (c) The amount of liquid in the food press. (d) The even distribution of sample on the walls so that there is no excess pressure on any one part of the samples. (e) The effect of pressure on the wooden piece of the apparatus. In this treatment it was desired to remove as much endosperm and aleurone as possible without loss of any pericarp. It was impossible, however, to remove satisfactorily all of the endosperm and aleurone from the pericarp in the food press. Next the pericarp was placed in a 500 cc. salt-mouth bottle containing approximately 20 medium sized ball mill pebbles and nearly full of water.

The ball mill consisted of a tumbling machine so constructed that the reagent bottles traveled end-wise through a radius of about 8 inches, at the rate of 41 R.P.M. In this operation the pebbles, falling from one end of the jar to the other during the grinding period served to loosen bits of remaining endosperm and aleurone layer from the pericarp. After grinding, the samples were washed with 4 or 5 portions of distilled water through a conical 8-mesh sieve. In this way all of the remaining particles other than pericarp were removed from the residue which was then washed free from the pebbles on a 14-mesh sieve, pressed into a flat metal pan and dried to constant weight along with the moisture samples. Official methods were used for moisture in the air-dry sample, ether extract, crude fiber and total nitrogen.

The hybrids and inbreds were classified into four classes as follows: tender, medium tender, tough and very tough. This was an arbitrary classification based, according to the judgment of the writers, on chewing tests or on the general reputation of the material in the canning and the seed trade. Corn is known to be tougher to chew as it matures. The same variety appears to be tougher at 66-68 per cent than at 70-72 per cent moisture. The increase in toughness is generally attributed to increased toughness of the pericarp or increase in the quantity of pericarp.

In table 1 the data show that an increase in maturity in the case of Minhybrid 202 was accompanied by an increase in the percentage of peri-

carp. On the other hand, a strain classified as tough might contain a high percentage of pericarp as shown by inbred 13, or a strain might be classed as medium tender and have a lower percentage of pericarp at 68 per cent moisture (old) as shown by inbred 1445.

TABLE 1. *The percentage of pericarp present in several sweet corn inbreds and hybrids in 1938 and 1939*

| | 1938 | | 1939 | |
|---|---|---|---|---|
| | Percentage | | Percentage | |
| *Tender* | Moisture | Pericarp oven-dry basis | Moisture | Pericarp oven-dry basis |
| 5. Ioana ..... | 71.1 | 5.6 | 70.8 | 4.3 |
| 13. Iogent 27 ..... | 73.1 | 6.8 | 67.8 | 5.2 |
| 15. Golden Cross Bantam | 70.7 | 4.4 | 74.6 | 4.8 |
| 17. Inbred 1627 ..... | 72.4 | 4.5 | 68.4 | 4.5 |
| *Medium tender* | | | | |
| 1. Minhybrid 201 ..... | 74.1 | 5.6 | 78.0 | 5.1 |
| 12. Iogent 12 ..... | 70.1 | 6.4 | 68.5 | 5.8 |
| 18. Inbred 1445 ..... | 68.0 | 5.8 | 68.2 | 5.3 |
| 8. Inbred 191 ..... | 69.8 | 5.6 | ... | .. |
| *Tough* | | | | |
| 2. Minhybrid 202 ..... | 74.6 | 5.3 | ... | .. |
| 3. Minhybrid 202 ..... | 72.2 | 6.1 | 72.9 | 6.3 |
| 4. Minhybrid 202 ..... | 66.4 | 7.1 | ... | .. |
| 6. Iogold 13 x 45 ..... | 73.7 | 6.1 | 75.7 | 6.4 |
| 11. Inbred 13 ..... | 75.0 | 6.4 | 77.1 | 6.0 |
| 14. Inbred 1214 ..... | 72.5 | 5.5 | ... | .. |
| *Very tough* | | | | |
| 10. Inbred T. P. ..... | 74.6 | 8.4 | 78.1 | 8.5 |
| Inbred 1620 ..... | 66.6 | 7.5 | 71.5 | 7.7 |

This method used for determining the amount of pericarp gives comparisons between strains irrespective of moisture content within the range when corn is commonly used for canning. The kernels of tender strains contain 4-5 per cent pericarp, medium tender from 5-6 per cent, tough from 6-7 per cent and very tough over 7 per cent on a dry weight basis. The 1939 results fit into this classification with one exception. The 1938 results were not quite so consistent, but the technique of sampling and separation of the pericarp had been improved in 1939. Tough and tender pericarp strains of sweet corn differ in the quantity of pericarp present.

## SUMMARY

1. A method is presented for separating the pericarp of sweet corn from the other tissues of the kernel at the edible stage.
2. Inbred and hybrid strains of sweet corn differ in the quantity of pericarp at the canning stage.
3. Strains classed as very tough on chewing contain from 50 to 100 per cent more pericarp than those classed as tender.

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# THE RESPONSE OF THE PLUM GROWN UNDER HILLCULTURE CONDITIONS TO MODIFICATIONS IN CULTURAL TREATMENT[1]

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Received June 7, 1940

Systematic hillculture under the Dutch name—bergculture—has been practiced in the Dutch East Indies for many years. When farming first began there, it was found that the heavy rains and the steep erosive soil made it necessary to use some other system than clean cultivation. It was considered so important to develop a better agricultural system that private land owners began experimenting with combining types of plants to keep the soil almost completely covered at all times. Terracing and furrowing methods were used to insure the conservation of soil and water.

In the United States the purpose of the experiments is to develop for steep eroded soils a rational system of erosion-control farming based on the use of superior plants, so managed as to improve the soil, conserve water, and produce permanent annual yields and reasonable profits.

In the extensive erosion area of southern Iowa and northern Missouri one of the immediate problems seemed to be the development of a cultural system based on the foregoing principles, for the utilization of a portion of the steep eroded land of the area to raise an adequate supply of fruit for local use. Of the tree fruits considered, the plum was one selected for experimentation because its flexibility in habitat requirements gave promise of adaptation to a wide range of modification in cultural treatment.

## MATERIALS AND METHODS

The plantings are located on the Hillculture Experimental Farm in Davis County in southeastern Iowa. The plums were planted on approximately five acres of land in three fields located on southeast, northeast, and east slopes of 10 to 30 per cent. The soil type at the top of each slope is Clinton and the remainder is Lindley. The farm had been abandoned because of excessive erosion, the depth of the remaining A horizon on the selected slopes varying from zero to ten inches.

The trees were planted at intervals of 13.2 feet on furrows on exact contours 10 feet to 20 feet apart. For each row two furrows were thrown down the slope with a tractor-drawn 16-inch plow. The lower furrow had a depth of approximately eight inches. The upper furrow, on top of which the trees were planted, was approximately four inches deep.

Before the furrows were made in the early spring of 1938 the plant cover of the slopes varied from a thin cover of bracted plantain and rag-

[1] Journal Paper No. J-758 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 582.

weed, through a mixture of bracted plantain, aster and goldenrod, to a medium dense stand of Canadian and Kentucky blue grass, sweet clover, and clover. At no time since the furrows were made has more than 10 per cent of the rainfall been lost from the slopes and for most of the time the entire precipitation of about 32 inches per year has been retained. During the two seasons of the experiment the original vegetation remaining between the rows of trees has materially increased in density. Plate I shows the general appearance and aspect of the northeast slope.

The plum varieties used were the Green Gage variety of *Prunus domestica* and the following Minnesota Agricultural Experiment Station improved varieties which have been produced from crosses between American and Asiatic plums: Monitor, Superior, Tonka, and Underwood. The trees were commercial stock raised at Shenandoah, Iowa, and were 11/16 inch, 5-7 feet, well branched trees, pruned to five 6-inch branches.

The trees were planted (April 18-22, 1938) in plots of three contour rows, each row containing 5 or 6 trees of a given variety. The three-row units vary on the slope from a length of two plots to a length of six plots. The location of the five varieties was randomized except that there were fewer plots of Green Gage and Tonka than of the other three varieties. Since the Minnesota varieties of plums are poor pollenizers, an adequate number of single pollenizer plums were put in at the end of the middle contour rows of the plots.

In contrast to the arrangement of the trees in plots of three by five or six trees, the treatments were modified row by row; each contour received the same treatment for its entire length. Each plot of 15 or 18 plants of the same variety had three different treatments and each complete treatment row covered from three to six varieties.

All of the treatments received at least minimum culture, which consisted of planting on the top of the upper furrow and hand hoeing during the growing season to a 1.5-foot radius. The treatments were as follows: (1) The original plant cover adjacent to the row was undisturbed for at least four feet with 3-foot plow strips on each side of the row for interplanting of strawberries, cane fruits, sweet potatoes, asparagus, etc.; (2) The original plant cover on each side of the original double furrow was disked twice each season; (3) The original plant cover on each side of the original double furrow was left undisturbed; (4) The soil in the bottom of an extra furrow, about one foot above the row, was loosened to a depth of 2.5 feet, comparable to the action of a 6-inch chisel plow; (5) Two extra furrows were plowed above the row of trees and kept cultivated; (6) The row of trees was plowed and cultivated once above and below for a distance of at least four feet and kept mulched with the exception of a small open furrow on each side; and (7) The row of trees was plowed and kept cultivated above and below for a distance of at least four feet on each side.

## RESPONSE OF THE TREES TO VARIATIONS IN TREATMENT

In spite of the fact that the varieties had been tested for conditions comparable to those of southern Iowa, there was a definite variety dif-

ference in survival and growth (table 1). At the end of the first growing season survival was satisfactory for all varieties except Tonka, which showed a survival of only 60 per cent.

The trees planted in unfurrowed rows for comparison with those planted on contour furrows had only minimum culture and were compared with the minimum culture treatments on the contour furrows. The trees on the contour furrows showed by their response that they had an advantage in growth. A properly designed experiment for the purpose of obtaining quantitative data on this phase of the problem was set up in May, 1939.

Differentiated response of the young plum trees to variations in cultural treatment during the first growing season was negligible. This was expected since the trees, which were three years old and sturdy, probably contained sufficient reserve materials to make adequate growth under a wide range of growing conditions. There seemed to be little difference in height and branch growth between those trees which were expanding chiefly at the expense of stored food reserves and those which were becoming well established and were producing adequate supplies of reserve materials.

TABLE 1. *Survival and growth response of the five plum varieties tested, 1938*

| Variety | Survival (percentage) | Length permanent branches per plant (inches) | Average height increase (inches) | Rank |
|---|---|---|---|---|
| Green Gage | 93 | 32 | 5.3 | 4 |
| Monitor | 95 | 72 | 7.3 | 3 |
| Superior | 100 | 128 | 11.4 | 1 |
| Tonka | 60 | 29 | 4.3 | 5 |
| Underwood | 98 | 86 | 7.9 | 2 |

Figure 1 shows the difference in average number per plant of permanent branches during the two years. In number of permanent branches formed the first year, there was no measurable difference which could be attributed to variations in treatments. However, an increase in total height growth per tree of the chisel-furrow and the complete cultivation treatments was evident during the first year of growth (figure 3) probably because response in height growth seems to follow improved conditions more closely than does response in number of branches or in total length of branches.

During the second growing season (figure 1) the average number of branches per plant increased for each treatment over that of the first season. The magnitude of the increase seems to be progressively greater with cultural methods from minimum to complete cultivation. It was noticed also that at the end of the second season the plants of a given treat-

ment were more nearly alike in size and habit of growth than at the end of the first season. This is probably attributable to the fact that the individual differences in food reserves of the trees when planted tended to be equalized during the two seasons by growth of the plants under similar cultural conditions.

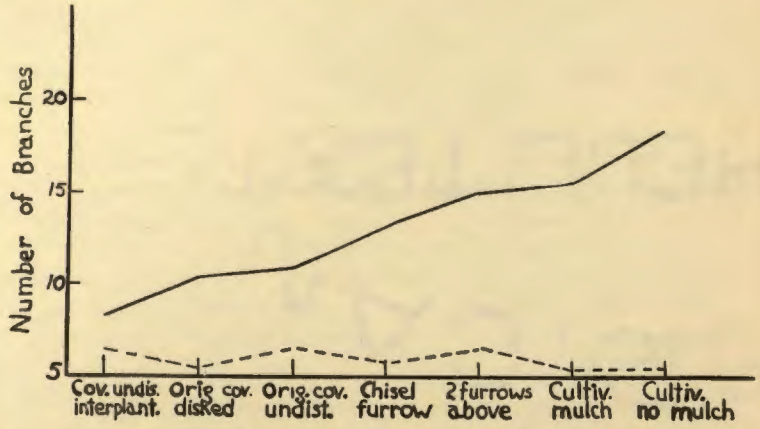


Fig. 1. Average number per plant of branches over 6 inches in length of all varieties on all slopes, 1938 ....., and 1939 .....

Three of the measurements of plant response used are compared in figure 2. The correspondence in the three measurements used to indicate response of the plants to different treatments is close enough that they might, in most cases, be used interchangeably. A graph made of the mean of the three curves (figure 2) may be used to good advantage as a curve of average plant response.

An average plant response curve of this type made from figure 2 shows that there is an increase in growth from left to right. However, there is little difference among the three minimum culture treatments on the extreme left. The competition with the original cover next to the trees seemed to be sufficient to suppress the growth of the trees, whether or not the cover was disked. Disking was not necessary to hold water on the slope since it was held by the furrows.

There is a definite increase in rate of growth from the three minimum culture treatments on the left (figure 2) to the three medium culture treatments, immediately to the right. These three treatments, the chisel furrow, two furrows kept cultivated above, and cultivation with mulch, seem to result in adequate growth response and also offer promise of adequate soil protection and soil building. Average response was higher for complete cultivation than for any of the other treatments, but soil losses into the bordering furrows and beyond make this method impracticable.

In figure 3 is shown the variation in response of the plums induced by the complex of factors represented by three different sites. While the average degree of slope varied little for the three sites, direction of slope and

general climatic factors were different. However, the greatest difference among the three sites was the degree of erosion and the density and vigor of growth of the original plant cover.

The A horizon of the soil on the northeast slope varied in depth from six to ten inches, which during the second year supported a dense growth

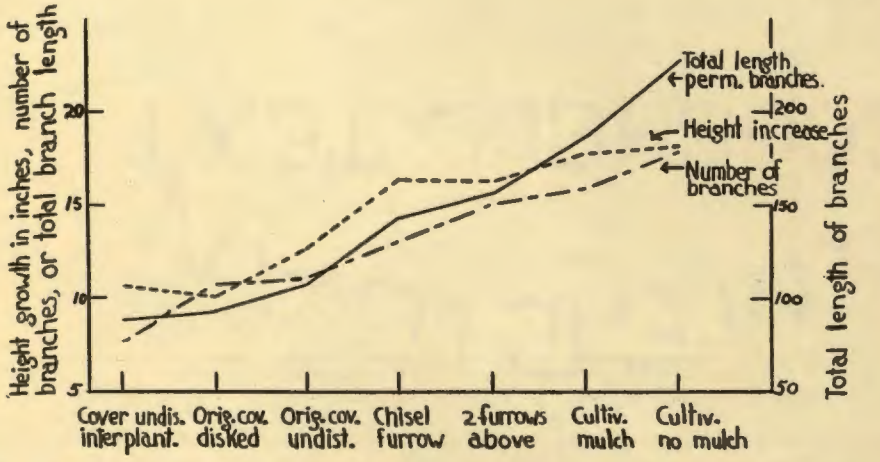


Fig. 2. Comparison of the response of the plum trees in terms of total length of permanent branches, increase in height, and total number of branches. Average of all trees on all sites. 1939.

of bluegrass, sweet clover, and red clover. In contrast, the A horizon of the southeast site was almost entirely removed except near the bottom. The vegetation was sparse and represented an early stage of the succession: the ragweed-bracted plantain stage. The soil of the east slope was about half way between the other two in degree of erosion, and with original cover of the late weed-early bluegrass stage.

The trees on the northeast slope showed poor height growth with minimum culture (figure 3) and improved very little with chisel-furrow treatment, but gave good results where the competition of the original cover was reduced by plowing and the trees were either mulched or kept cultivated. Approximately the same results were obtained with minimum culture on the east slope, but the fertility of the soil was sufficiently low that release from competition by mulching and clean cultivation resulted in less tree growth than on the fertile soil of the northeast slope. On the southeast slope the trees did better under minimum culture where the water was held by furrows and the thin layer of top soil was not disturbed by cultivation. The poor response to plowing and mulching and to cultivation can probably be attributed to the low fertility of the soil and to the fact that the turning over and loosening of the small quantity of top soil resulted in excessive leaching if not to loss of fertilty by erosion. There was also a greater increase in the response in total length of perma-

nent branches of cultivated trees on the east and northeast slopes than on the southeast slope.

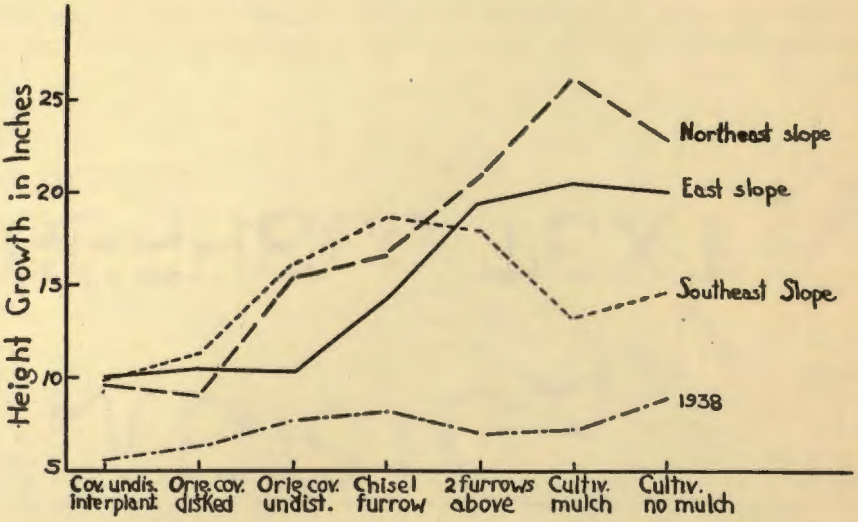


Fig. 3. Average height growth per plant of all varieties on each of the three sites for the growing season of 1939 compared with the average for all varieties and all sites, 1938.

## SUMMARY

1. Five selected varieties of commercial plums were planted in 1938 on contour furrows on steep eroded soil of the Lindley and Clinton types in southern Iowa.
2. The variation in the response of the trees to seven different cultural treatments on the contour furrows and to minimum culture without furrowing was greater the second year than the first.
3. Three different measurements of plant response seem to give about the same differences between treatments: (1) total length of permanent branches; (2) height increase; (3) number of branches.
4. Medium cultural treatments seemed to be superior to minimum culture in growth and establishment of the trees.
5. Complete cultivation resulted in some increase in growth but seems impracticable on the steep erosive soils used in the experiment.
6. Fertility, degree of erosion, aspect and degree of slope, and competition of the original plant cover are all important in the selection of the best cultural treatment to use.

## PLATE I

Commercial plums planted on the contour of a northeast slope in April, 1938. July, 1939.

PLATE I

# MOTILITY OF THE EXCISED FORE-GUT OF PERIPLANETA AMERICANA (ORTHOPTERA) IN VARIOUS SALT SOLUTIONS

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Received June 13, 1940

Physiological salt solutions have been perfected for many different animals, but there is little available information about balanced salt solutions for insects. A fluid which will maintain insect tissues in a satisfactory condition for several hours should prove useful in the furtherance of both insect physiological and toxicological knowledge. This paper reports the results of muscular activity in various salt solutions registered kymographically by 450 excised crops from the cockroach, *Periplaneta americana*. The series of experiments described here is the first step of an attempt to produce an insect Ringer's solution for the American roach. The investigations will be continued in order to determine the specific effect of the separate ions.

Several insect physiological solutions have been tried. In 1917, Brocher (2) investigated heart contractions of *Dytiscus marginalis* in a balanced salt solution, but neglected to report its composition. In the same year, Glaser (5) grew hemolymph cells from larvae of *Malascoma americanum, Cirphis unipuncta, Laphygma frugyperda,* and *Porthetria dispar* in a Locke solution which he stated was an isotonic salt mixture. In a study of the movement of malphigian tubules from *Drosophila funebris* and *Calliphora erythrocephala,* Eastham (4) employed NaCl solutions of several concentrations. Hobson (6), in 1928, reported an extensive study of excised crop and esophageal contractions from *Dytiscus marginalis* during which he used a simple modified Ringer mixture.

In 1928, Levy (7) attempted to find an adequate solution in which to study heart preparations of *Phormia regina* and *Calliphora erythrocephala.* He made a stock solution (108 grams of NaCl, 8.5 grams of KCl, 5.5 grams of $CaCl_2$, 2.0 grams of $NaHCO_3$, and 0.1 gram of $NaH_2PO_4$ in 10 liters of solution), tried various dilutions, and reported a twelfth dilution as giving the best results. Yeager and co-workers (14, 15, 16) have used a slightly modified Levy's solution when experimenting with contractions of heart and malphigian tubules from *Periplaneta americana, Prodenia eridania,* and *Blatta orientalis.*

Slifer (9), in 1934, used a solution, which had previously been reported by Belar (1), as a medium in which embryos of the grasshopper *Melanoplus differentialis,* were grown. Belar had originally devised this solution for studies of mitoses in excised testes from *Chorhippus lineatus.* While experimenting with the effects of certain drugs on crop contractions of *Dytiscus marginalis,* TenCate (11), in 1929, used a 0.95 per cent NaCl solution.

Table 1 gives a summary of the concentrations of the constituents in the solutions listed in the foregoing.

## METHODS AND MATERIALS

The apparatus for maintaining and recording crop activity in experimental salt solutions is diagramed in figure 1, and described below. The solution to be tested was placed in an aspirator bottle (B) where oxygen was bubbled through it before and during each trial. The fluid ran through inlet tube (I) into the crop chamber which was made of a large glass tube (T). Cork (C) sealed with paraffin closed the chamber at one end. Clamp (Cl) closed off the outlet tube (O) so that the solution was drained out through overflow tube (OO). Rate of flow was regulated by stop cock (S). The excised crop (Cr) was suspended by two silk threads (ST) between a wire hook (H) and wax (W) on the end of the capillary glass recording lever (RL). Contractions were recorded on a kymograph (K) which stood upon a leveling table. (Figure 1.)

To remove the crop, the roach was first inactivated with ether vapors. After the legs had been removed close to the body wall, the specimen was fastened, ventral side up, in a small wax-bottom dissecting pan where it was immersed in some of the particular solution to be tested. The ventral body wall was carefully removed so that the entire digestive tract, from esophagus to hind-gut, was exposed. After cutting the remaining muscle and tracheal connections to the head and the crop, the fore-gut was severed from the hind-gut just posterior to the gizzard and was removed from the body. By means of a slip knot, one short thread was tied to the gut in the region of the ventriculus. No perceptible difference in crop activity was evident when the attachment was either immediately anterior or immediately posterior to the gizzard. Another longer thread was attached to the antennae. With a loop at the loose end, the shorter thread was fastened to a hook in the crop chamber. The longer thread was attached to the recording lever adjusted in length so that the actual amplitude of contraction was magnified seven times. A flow of physiological solution was maintained through the chamber at a rate of approximately 500 cc. per hour. Oxygen was bubbled through the solution in the stock bottle for the duration of each trial. All this work was done at room temperature, between 20°-26°C.

Each preparation was maintained until failure of the crop to contract terminated the experiment. Since the kymograph drum made one revolution in 16 hours, the duration of activity could be calculated in hours. Measures of amplitude were made in centimeters at intervals of one centimeter (or about every 20 minutes) throughout the time of activity. From these figures the mean amplitude was determined. Multiplication of the average amplitude and the duration of contractions yielded a product which was used as a measure of the activity of the individual crop and, consequently, as a criterion of the adequacy of a given solution. Since it was necessary to use a very slowly revolving kymograph drum, it was impossible to measure frequency of contraction in the present work.

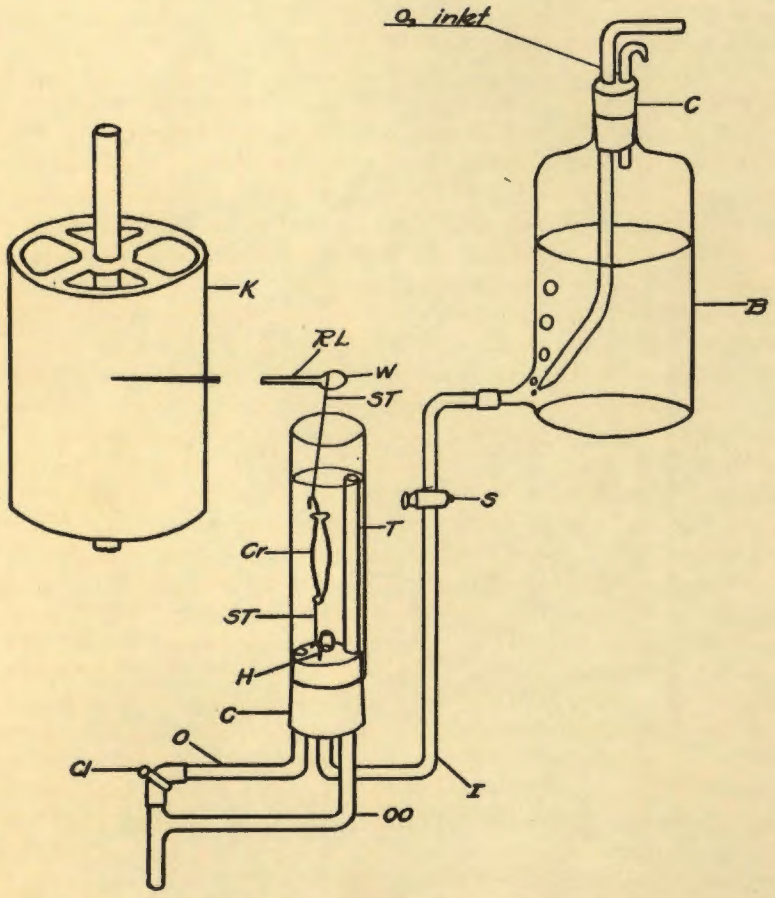


Fig. 1. Diagram of an apparatus for recording movement of the excised fore-gut of the American cockroach. (See text for complete description.)

TABLE 1. *Insect physiological solutions*

| Author | Date | NaCl g/l | KCl g/l | $CaCl_2$ g/l | $NaHCO_3$ g/l | $NaH_2PO_4$ g/l | $MgCl_2$ g/l | Glucose | Peptone |
|---|---|---|---|---|---|---|---|---|---|
| Brocher | 1917 | | (not reported) | | | | | | |
| Glaser | 1917 | 9.00 | 0.42 | 0.25 | 0.20 | | | 2.50 | 2.00 |
| Eastham | 1925 | 7.50 | | | | | | | |
| Eastham | 1925 | 6.00 | | | | | | | |
| Hobson | 1928 | 9.22 | 0.22 | 0.22 | | | | | |
| Levy | 1928 | 9.00 | 0.71 | 0.46 | 0.17 | 0.01 | | | |
| TenCate | 1929 | 9.50 | | | | | | | |
| Slifer (Belar) | 1934 | 9.00 | 0.20 | 0.20 | 0.20 | | | | |
| Yeager and Hager | 1935 | 9.82 | 0.77 | 0.50 | 0.18 | 0.01 | | 1.00 | |
| Yeager, Hager and Straley | 1935 | 9.00 | 0.70 | 0.46 | | few drops | | 1.00 | |
| Yeager | 1939 | 10.93 | 1.57 | 0.85 | | | 0.17 | | |
| Yeager | 1939 | 11.78 | 0.92 | 0.66 | | | | | |

(See Plate I.) Therefore, only amplitude and length of time of activity were used to calculate the activity product, expressed in centimeter-hours.

Ten crops, five from males and five from females, were tested in each solution. Preliminary determinations were made with the solutions as recommended by Slifer (9), TenCate (11), Hobson (6), Yeager (14), and Glaser (5). Using the best results from these solutions as a basis for composition of the various experimental solutions, NaCl and $NaHCO_3$ concentrations were established at 9.75 and 0.19 gram per liter of solution, respectively. The KCl and $CaCl_2$ concentrations were varied from 0.0 to 1.19 grams per liter for the former salt and 0.0 to 0.89 gram per liter for $CaCl_2$. Three of these latter mixtures, with the addition of 0.19 gram of $NaHCO_3$ per liter, were used for additional experiments. For each of these three KCl-$CaCl_2$ ratios (0.67-0.33, 0.45-0.50, and 0.22-0.22 gram per liter) six NaCl levels (4.87, 9.75, 11.31, 12.87, 14.63, and 16.19 grams per liter) were tested in an attempt to determine the optimum NaCl concentration.

The experimental animals were adult specimens of the American roach, *Periplaneta americana,* reared in the laboratory at room temperature, and fed a diet of whole wheat bread and banana, supplemented occasionally with hamburger.

## RESULTS AND DISCUSSION

Yeager (12) observed three types of proventricular movement in intact *Periplaneta fuliginosa:* peristaltic, antiperistaltic, and contractions involving the posterior third of the crop. These same types of movement were readily observed in the excised fore-gut of the American roach. Often peristaltic or antiperistaltic waves passed down or up the crop without visible effect upon the recording lever. The pulling contractions of the posterior third of the crop accounted for the majority of the recorded movements.

Results from each lot of ten crops in the 43 solutions are tabulated in table 2. The following information is listed for each solution: (1) concentrations of NaCl, KCl, and $CaCl_2$ in grams per liter; (2) the KCl/$CaCl_2$ ratio; (3) the average amplitude, in centimeters, of contraction for the ten crops in each solution; (4) the average length of time, in hours, of activity for the ten crops tested in each mixture; (5) the average activity products in centimeter-hours for crops from males and females; and (6) the average activity product of all crops in each solution. All solutions contained approximately 0.19 grams of $NaHCO_3$ per liter of solution, with the exception of Hobson's and TenCate's which contain none . (Table 2.)

Examination of the survey data from solutions containing from 9.00 to 10.00 grams of NaCl per liter showed that only five (22, 23, 20, Belar's, and Hobson's) produced activity products which indicated that they were at all satisfactory, both in regard to average amplitude and length of time to activity (see table 2). Of these, solution number 22 produced

TABLE 2. *Rating of solutions according to average activity products from ten crops**

| Solution | NaCl | KCl | $CaCl_2$ | KCl / $CaCl_2$ | Ampl. (cm.) | Time (hr.) | Activity Products ♂ | ♀ | Av. |
|---|---|---|---|---|---|---|---|---|---|
| 25 | 14.63 | 0.45 | 0.50 | 0.90 | 0.160 | 13.42 | 2.628 | 1.886 | 2.257 |
| 29 | 14.63 | 0.67 | 0.33 | 2.03 | 0.099 | 9.04 | 0.693 | 1.488 | 1.091 |
| 32 | 16.19 | 0.45 | 0.50 | 0.90 | 0.057 | 13.13 | 1.062 | 0.719 | 0.891 |
| 22 | 9.75 | 0.67 | 0.33 | 2.03 | 0.071 | 8.41 | 1.312 | 0.459 | 0.885 |
| 31 | 12.87 | 0.45 | 0.50 | 0.90 | 0.080 | 9.91 | 0.689 | 0.750 | 0.719 |
| 38 | 16.19 | 0.22 | 0.22 | 1.00 | 0.056 | 9.32 | 1.197 | 0.146 | 0.671 |
| 37 | 12.87 | 0.22 | 0.22 | 1.00 | 0.067 | 8.87 | 0.923 | 0.315 | 0.619 |
| Belar | 9.00 | 0.20 | 0.20 | 1.00 | 0.055 | 9.07 | 0.921 | 0.253 | 0.587 |
| 23 | 9.75 | 0.45 | 0.50 | 0.90 | 0.060 | 7.39 | 0.904 | 0.110 | 0.507 |
| 34 | 12.87 | 0.67 | 0.33 | 2.03 | 0.045 | 9.51 | 0.528 | 0.420 | 0.474 |
| 26 | 14.63 | 0.22 | 0.22 | 1.00 | 0.070 | 7.76 | 0.311 | 0.514 | 0.412 |
| 35 | 16.19 | 0.67 | 0.33 | 2.03 | 0.042 | 7.13 | 0.321 | 0.478 | 0.400 |
| 30 | 11.31 | 0.45 | 0.50 | 0.90 | 0.039 | 7.18 | 0.398 | 0.400 | 0.399 |
| 20 | 9.75 | 0.22 | 0.33 | 0.67 | 0.049 | 8.91 | 0.449 | 0.333 | 0.391 |
| Hobson | 9.22 | 0.22 | 0.22 | 1.00 | 0.048 | 8.22 | 0.365 | 0.405 | 0.385 |
| 3 | 9.75 | 0.89 | 0.67 | 1.33 | 0.035 | 9.16 | 0.415 | 0.195 | 0.305 |
| 36 | 11.31 | 0.22 | 0.22 | 1.00 | 0.038 | 5.97 | 0.154 | 0.436 | 0.295 |
| Yeager | 9.82 | 0.77 | 0.50 | 1.54 | 0.036 | 8.46 | 0.243 | 0.344 | 0.294 |
| 21 | 9.75 | 0.45 | 0.17 | 2.65 | 0.046 | 4.26 | 0.421 | 0.158 | 0.290 |
| 4 | 9.75 | 0.89 | 0.89 | 1.00 | 0.040 | 5.32 | 0.540 | 0.022 | 0.281 |
| 6 | 9.75 | 0.45 | 0.67 | 0.67 | 0.050 | 6.30 | 0.371 | 0.154 | 0.262 |
| 16 | 9.75 | 0.45 | 0.33 | 1.36 | 0.048 | 5.19 | 0.462 | 0.043 | 0.252 |
| 15 | 9.75 | 0.22 | 0.50 | 0.44 | 0.044 | 3.56 | 0.276 | 0.173 | 0.225 |
| 5 | 9.75 | 0.22 | 0.67 | 0.33 | 0.028 | 5.23 | 0.339 | 0.010 | 0.174 |
| 8 | 9.75 | 1.19 | 0.89 | 1.34 | 0.026 | 3.01 | 0.319 | 0.016 | 0.168 |
| 2 | 9.75 | 0.89 | 0.33 | 2.70 | 0.018 | 4.53 | 0.198 | 0.048 | 0.123 |
| 12 | 9.75 | 0.67 | 0.67 | 1.00 | 0.037 | 2.48 | 0.211 | 0.009 | 0.110 |
| TenCate | 9.50 | 0.00 | 0.00 | 0.00 | 0.069 | 1.54 | 0.036 | 0.134 | 0.085 |
| 11 | 9.75 | 0.00 | 0.44 | 0.00 | 0.022 | 2.61 | 0.075 | 0.022 | 0.049 |
| Glaser | 9.00 | 0.25 | 0.42 | 0.60 | 0.024 | 2.47 | 0.033 | 0.063 | 0.048 |
| 1 | 9.75 | 0.89 | 0.17 | 5.24 | 0.042 | 2.04 | 0.022 | 0.048 | 0.036 |
| 24 | 4.87 | 0.45 | 0.50 | 0.90 | 0.044 | 2.20 | 0.044 | 0.011 | 0.028 |
| 9 | 9.75 | 0.45 | 0.00 | 0.00 | 0.037 | 1.50 | 0.016 | 0.040 | 0.028 |
| 33 | 11.31 | 0.67 | 0.33 | 2.03 | 0.015 | 1.20 | 0.042 | 0.006 | 0.024 |
| 10 | 9.75 | 0.00 | 0.22 | 0.00 | 0.033 | 0.51 | 0.020 | 0.009 | 0.015 |
| 7 | 9.75 | 1.19 | 0.67 | 1.78 | 0.017 | 0.34 | 0.009 | 0.002 | 0.006 |
| 17 | 9.75 | 0.67 | 0.17 | 3.94 | 0.016 | 0.25 | 0.008 | 0.004 | 0.006 |
| 14 | 9.75 | 0.00 | 0.67 | 0.00 | 0.018 | 0.45 | 0.007 | 0.003 | 0.005 |
| 13 | 9.75 | 0.00 | 0.89 | 0.00 | 0.037 | 0.17 | 0.002 | 0.009 | 0.005 |
| 28 | 4.87 | 0.67 | 0.33 | 2.03 | 0.009 | 0.37 | 0.003 | 0.004 | 0.004 |
| 27 | 4.87 | 0.22 | 0.22 | 1.00 | 0.004 | 0.69 | 0.005 | 0.002 | 0.004 |
| 18 | 9.75 | 0.89 | 0.00 | 0.00 | 0.006 | 0.68 | 0.001 | 0.004 | 0.003 |
| 19 | 9.75 | 1.19 | 0.00 | 0.00 | 0.009 | 0.11 | 0.003 | 0.001 | 0.002 |

*Figures for amplitude of contraction (Ampl.) and for hours of activity (Time) are averages based on tracings from 10 crops tested in each solution.

the highest average activity product, and maintained contractions, in one instance, for more than 26 hours. Although several exceptions were present, it appeared also that, in general, the concentrations of both KCl

and $CaCl_2$ should be less than 1.0 gram per liter of solution and that the $KCl/CaCl_2$ ratio must be within the range of 0.7 to 2.0. Investigations to determine optimum concentrations for these salts are now in progress.

When the various NaCl percentages were tested with the three $KCl$-$CaCl_2$ concentrations (see methods for details), the following results were obtained. With only 4.87 grams of NaCl, the activity products dropped to almost nothing, as with solutions 24, 27, and 28 (0.028, 0.004, 0.004). Apparently the tissue could not maintain contractions with this lowered content of NaCl. However, when the amount of NaCl was increased, greater activity resulted. Ranked according to activity products, nine of the first twelve solutions had NaCl concentrations higher than 12.87 grams per liter. Six of these nine maintained an average activity time of more than nine hours (see table 2). Not only was the length of time of activity longer when the NaCl was increased, but also the average amplitude was higher. Among the high NaCl concentrations are average amplitude readings of 0.160, 0.099, and 0.080 centimeter. The lower NaCl levels approached these values in only one instance, number 22, with 9.75 grams per liter which gave an average ampliture of 0.71 centimeter.

Plate I consists of kymograph records which indicate the difference in general appearance between tracings made by crops in various salt mixtures. *A* and *F* were made in solution 25 which has given the best results (average activity product of 2.3) in tests so far conducted; *B*, *C*, *D*, and *E* are recordings of crop activity in solutions 21, 20, 23, and 20, respectively. These latter salt mixtures gave average activity products approximately one-fourth to one-seventh of that of number 25. Poorer solutions would give records with only a few erratic contractions. Figure 2 also demonstrates that baselines in records from certain solutions are quite irregular, as in *B*, *C*, *D*, and *E*. This variability was particularly evident during the first hour or hour and a half of a crop's subjection to solutions with less than 10 grams of NaCl per liter, which was the case in the mixtures from which these records resulted. Often many minutes passed before contractions started. In general, activity in the solutions with high NaCl (12.87 grams or more) started immediately with high amplitude and a stable baseline, (*A* and *F*), both of which continued throughout the major portion of the time of activity. These conditions were especially evident in solution number 25, which contains 14.63 grams of NaCl, 0.45 grams of KCl, 0.50 gram of $CaCl_2$, and 0.19 grams of $NaHCO_3$. (Plate I.)

A more thorough investigation of higher levels of NaCl, with other $KCl$-$CaCl_2$ concentrations, are now in progress to ascertain a mixture of optimum salt concentrations for the type of experiment described. The results from the above trials seem to indicate that greater amounts of NaCl, than those which have been reported in the literature (see table 1), are beneficial in maintaining high amplitude and longevity of muscular contraction in the fore-gut of the American roach. Whether the possibility that such a solution will be good for all types of roach tissue, or for

materials from other insect species, has not yet been explored. It is worthy of note, however, that Yeager (13), in 1939, found that increasing the NaCl concentrations in his physiological solutions to 10.93 and 11.78 grams per liter gave better results when studying electrical stimulation of the dorsal vessel in the cockroach, *Periplaneta americana*. Previously he had used 9.0 or 9.82 grams of NaCl per 1000 cc.

The supposed role of the sodium ion is that of an osmotic regulator and a stimulatory ion. Cole and co-workers (3) found, while investigating heart contractions in the crayfish, that high concentrations of the sodium ion alone stimulated the heart to greater frequency, but proved to be toxic in that the longevity of the tissue was materially reduced. Also, according to Rogers (8, pp. 156), "it (sodium) exerts in many cases a distinct poisonous effect, acting particularly upon the cell membranes." Thus it appears that although an increase in the sodium content may cause hyperactivity, this increased response may last for only a short time, and over long periods of exposure, high sodium ion concentrations are detrimental. It seems, therefore, that a higher level of NaCl may be beneficial in producing an increased activity if it is maintained below a point where the ion exerts a toxic effect; and it appears from the foregoing experiments that a higher NaCl concentration than has so far been recommended in insect salines does have definite merit in maintaining the activity of the isolated fore-gut of the roach.

During the course of the present investigations a distinct difference was observed in both the appearance and the activity of the crops from male and female roaches. When analyzed statistically, there was a significant difference in the amount of food present in the crop. The crops from males were definitely more active and had food present in only a small number of cases. The actual presence of food, however, did not seem to be correlated with hyper- or hypo-activity of the crop. No attempt has been made to regulate the period of feeding prior to experimentation, but both males and females had equal access to the same kind of food. At present no adequate explanation can be advanced. However, Snipes and Tauber (10) found that, while not significantly different, the egestion time of female specimens of *Periplaneta americana* was slower than that of males (19.6 hours for males; 21.4 for females). Therefore, it appears that the muscular activity of the crop and digestive tract in the male American roach is greater than that of the female.

## SUMMARY

1. A method for recording and measuring the muscular activity of the excised fore-gut from the American roach is described. See figure 1.

2. Formulae for insect physiological salines which have been published were tested and found not to maintain activity as well as some other solutions which were devised during the course of the present experiments. See table 1. Published formulae gave activity products

which ranged from 0.048 to 0.587; the best solution tested in the present series by the writers gave an average activity product of 2.257.

3. Several concentrations of KCl (0.20 to 0.89 gram per liter) and of $CaCl_2$ (0.20 to 0.67 gram per liter) will maintain contractions of the excised crop, over a period averaging from 9 to 13 hours, when combined with the correct amount of NaCl, usually from about 10 to 15 grams per liter. The maximum length of activity so far obtained from any single crop was over 26 hours in solution number 22 which contained 9.75 grams of NaCl. (In general, however, results from 22 were not as good as from other solutions with higher NaCl content.)

4. Sodium chloride in concentrations of 12.87 to 16.19 grams per liter of solution, when combined with certain KCl-$CaCl_2$ ratios, appears to be beneficial by increasing the amplitude of contraction and the longevity of the excised tissue.

5. Solution 25 (14.63 grams of NaCl, 0.45 gram of KCl, 0.50 gram of $CaCl_2$, and 0.19 gram of $NaHCO_3$ per liter) produced an activity product twice as high as the next best solution and maintained crop activity for an average of more than 13 hours. Minimum duration was 3.7 hours; maximum, 25.3 hours. This mixture has not been tested on other tissues or under other conditions.

6. The crops from male roaches produced significantly higher activity products than those from females. Food was present in the fore-gut of the females more often than in the males.

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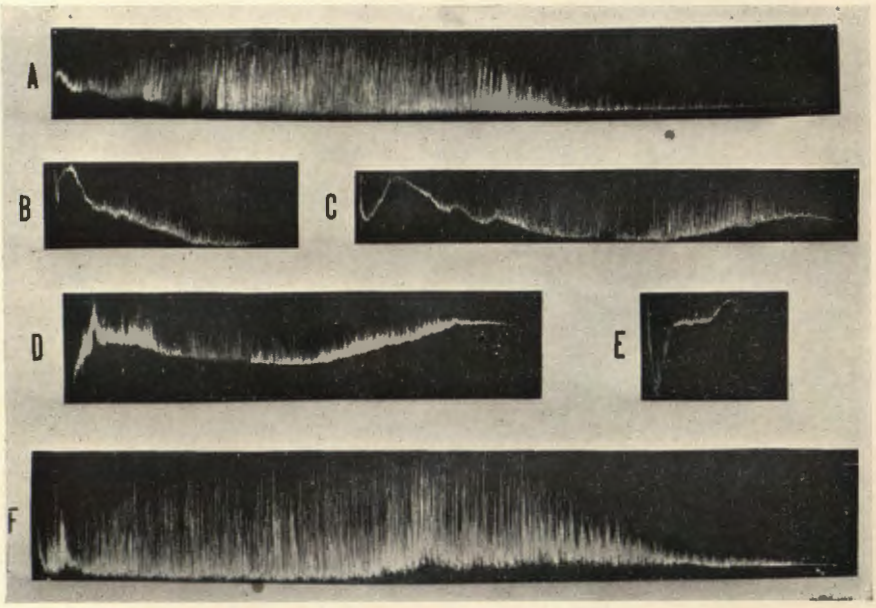
## PLATE I

Kymograph records of isolated crop activity. *A* and *F* were made in the best solution so far devised (number 25). *B*, *C*, *D*, and *E* are records from poorer solutions (number 21, 20, 23, and 20, respectively) and show the irregular baseline, erratic muscular response, and delayed beginning of contractions produced in such solutions. (See Table 2 for composition and activity products of these salt mixtures.)

PLATE I



# EFFECT OF ETHER ON THE TOXICITY OF CERTAIN FUMIGANTS TO THE CONFUSED FLOUR BEETLE, TRIBOLIUM CONFUSUM DUVAL[1]

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Received June 14, 1940

The study of synergism and antagonism in fumigant mixtures is a new and little-known field although these effects have been more thoroughly investigated in the field of local and general anesthesia. Synergism is defined broadly as "cooperation among several component parts" and in a toxicological sense may be said to be a lethal effect greater than additive. Antagonism, conversely, is a lethal effect less than additive.

The mechanism through which synergism or antagonism is obtained is not thoroughly understood. Bancroft and Richter (1931) explain synergistic action as that following the displacement of a drug by another drug which is adsorbed onto the same substrate. This displacement in effect increases the active or effective concentration of the primary drug and thus increases its toxicity in the cell. Bancroft and Richter (1931) state that antagonism occurs when the colloids of a cell are reversibly coagulated and the coagulating agent is replaced by a substance of weaker flocculation capacity at the given concentration. In this event the biocolloids are again peptized by the electrolytes of the cell.

The toxicity of ether to various insects has been investigated by a number of workers. McClintock, Hamilton and Lowe (1911) reported that carbon disulfide was apparently four times as toxic as ether to the housefly (*Musca domestica* L.), and that ether and chloroform were equal in their effect on houseflies. Shafer (1915) found that ether absorbed by an adult beetle (*Passalus cornutus*) did not interfere with the activity of the catalase present. Holt (1916) showed that carbon disulfide is about four times as toxic as ether to the cockroach. Moore (1917) states that carbon disulfide is roughly fifteen times as toxic as ether to houseflies. He found also that carbon tetrachloride was roughly twenty-seven times as toxic as ether to this insect.

Many other investigators have studied the effects of ether on insects. Among these are Moore and Graham (1918), Roark and Cotton (1929) and others. All have agreed that ether possesses low toxicity toward insects.

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[1] Journal Paper No. J-767 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 137.

[2] The writer gratefully acknowledges the inspiration and guidance of Dr. C. H. Richardson during the course of this research.

Carbon disulfide has been used as an insect fumigant since 1854, according to Simmons and Ellington (1926).

Carbon tetrachloride has been used as a fumigant only since about 1907, when Britton (1907, 1908a, 1908b) investigated the possibilities of this compound as a fumigant for nursery stock. Both of these compounds have been thoroughly tested by many competent workers on a wide variety of insects.

Ethyl acetate was recommended as a fumigant in combination with carbon tetrachloride by Back and Cotton (1925) It was later found that this material, although relatively non-inflammable, left an objectionable odor in fumigated grain. Shepard, Lindgren and Thomas (1937) state that a non-inflammable mixture of ethyl acetate and carbon tetrachloride is of low toxicity to stored-grain insects.

Neifert, et al. (1925) used a number of gas mixtures against adults of some of the more important grain-infesting insects. The authors state that less toxic substances in these mixtures were used principally as diluents.

Hazelhoff's (1928) experiments led him to make the suggestion that the addition of carbon dioxide to hydrogen cyanide, carbon disulfide and other fumigants would increase the toxicity of these compounds by increasing the rate of penetration of the gas.

Cotton and Roark (1927, 1928) and Roark and Cotton (1928) tested a number of gas mixtures and recommended a mixture containing three volumes of ethylene dichloride and one volume of carbon tetrachloride. This mixture was non-burnable and non-explosive.

Cotton and Young (1929) and Cotton (1930) showed that carbon dioxide increased the toxicity of given concentrations of carbon disulfide and chloropicrin to a marked degree. Back, Cotton and Ellington (1930) found that the addition of carbon dioxide greatly increased the efficiency of ethylene oxide as a fumigant.

Cotton (1932) found that carbon dioxide gave a maximum increase in toxicity to adults of *T. confusum* at concentrations of 37 lb. per 1000 cu. ft. with chloropicrin, 15 lb. per 1000 cu. ft. with carbon disulfide and 30 lb. per 1000 cu. ft. with ethylene oxide. When more than these amounts of carbon dioxide were added, the toxicity decreased.

Shepard and Lindgren (1934) give data on the relative toxicities of ethylene dichloride, propylene dichloride and their 75 per cent mixtures with carbon tetrachloride which show definite antagonism between these compounds and carbon tetrachloride.

Cupples, Yust and Hiley (1936) placed ether-HCN and carbon tetrachloride-HCN in the group of mixtures showing slight or no toxicity to red scale, while a carbon disulfide-HCN mixture was moderately toxic.

Cotton (1938) recommends the use of mixed gases for the control of insects attacking grain in farm storage. He states that mixtures of carbon disulfide with carbon tetrachloride and sulfur dioxide, to reduce the fire hazard, are now commercially available.

## EXPERIMENTAL METHOD

The insects used in these experiments were adults of the confused flour beetle (*Tribolium confusum* Duval). They were reared at 30° C. and a relative humidity of 60-70 per cent on white flour to which dried powdered yeast was added. Only beetles between 2 and 6 weeks of age were used in tests. During these experiments none of the many unfumigated check insects died, so it is apparent that natural mortality is a negligible factor within this age group.

The fumigation chambers employed in these experiments were balloon flasks of approximately 5.5 liters capacity. The apparatus was slightly modified from that used by Jones (1933) since all of the fumigants were introduced as liquids by means of burettes graduated to 0.01 cc.

From 30 to 50 adult beetles, selected at random from a large group, were used in each experiment. These were placed in glass cylinders, open at the top and closed at the bottom by one layer of cheesecloth, after which the cylinders were suspended from hooks in the tops of the flasks. The flasks were partially evacuated and the liquid fumigant introduced. The flasks were then placed at 30°±0.5°C. and returned to atmospheric pressure as soon as the liquid had evaporated. The beetles were exposed to the fumigant for a period of 2 hours, after which they were placed on clean flour at 30°C.

The temperature of 30°C. was used in order that the gases might be employed at higher activities than at 25°C. The exposure time of 2 hours was selected solely because of convenience, since a greater amount of data could be obtained in a given length of time.

Mortality counts were made at the end of 24, 48 and 96 hours. As stated by Shepard, Lindgren and Thomas (1937) and shown by Hamlin and Reed (1927, 1928) a serious source of variation lies in the difficulty of determining a sharp endpoint. In these experiments the final counts were made at the end of 96 hours. At that time the beetles were placed in one of three categories: alive, paralyzed in one or more pairs of legs, and dead. After exposure to ether and carbon disulfide, the beetles so injured by the fumigant that they were paralyzed at the end of 96 hours generally failed to recover. When these fumigants were employed, both paralyzed and dead beetles were used in determining the total mortality. With carbon tetrachloride, however, it was found that beetles completely paralyzed in as many as two pairs of legs might live for as long as a month in that condition and produce eggs. Here, only the number of beetles actually dead was used in determining the total mortality. The end point with ethyl acetate was quite sharp, and there were only minor changes in mortality after the 24-hour count. No great amount of paralysis was noted from this compound and only the number actually dead was used in determining the final mortality at 96 hours.

The data obtained from these experiments were plotted and analyzed statistically according to the method given by Bliss (1935). By using this method it is possible to transform the usual sigmoid dosage-mortality curve into a straight-line regression. The chi-square test may be applied to this regression line in order to determine the goodness-of-fit of the data. In addition, the limits of error may be calculated such that 95 per cent of the observations should fall within given areas or zones along the line. These limits of error were used in determining the antagonistic or synergistic effect of added toxic substances.

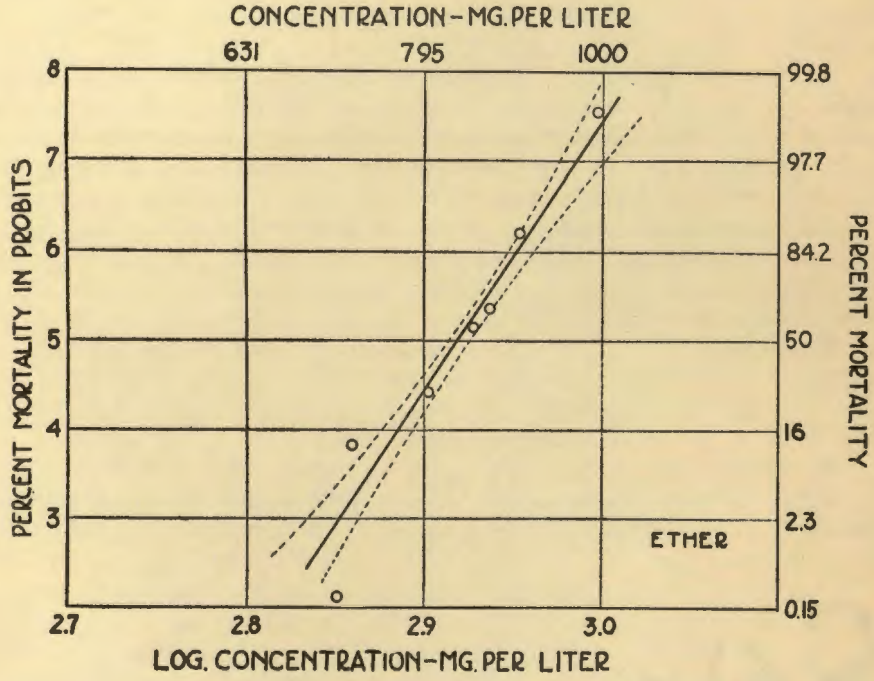


FIG. 1. Toxicity of ether to *Tribolium confusum* Duval.

The first series of experiments was concerned with the determination of the 2-hour median lethal concentrations of ether, carbon disulfide, carbon tetrachloride and ethyl acetate. Since the dosage-mortality relationship of ether to *Tribolium confusum* has not been reported previously, this curve was established rather carefully through a large number of experiments. Many previous workers have determined the effects of carbon disulfide, carbon tetrachloride and ethyl acetate on the confused flour beetle at a 5-hour exposure and 25°C. Shepard, Lindgren and Thomas (1937) also record the concentration of carbon disulfide and car-

bon tetrachloride killing 50 per cent and 99 per cent in 5 hours at 30°C. For that reason less time was spent in the determination of these curves.

Following these tests, the effect of the addition of sublethal concentrations of ether to the median lethal concentration of each of the other gases was determined. It was felt that any synergistic or antagonistic effect of ether upon the other gases would appear more strongly under such conditions.

## RESULTS

### A. TOXICITY OF FUMIGANTS ALONE

The 2-hour dosage-mortality curves at 30°C. for ether, carbon disulfide, carbon tetrachloride and ethyl acetate are shown in figures 1, 2, 3, and 4. The 2-hour median lethal concentrations and the concentrations killing 99 per cent of the insects are given in table 1.

TABLE 1. *Median lethal concentrations of fumigants against Tribolium confusum Duval*

| Fumigant | No. of insects tested | Concentration, in mg. per liter, required to kill— | |
|---|---|---|---|
| | | 50 per cent | 99 per cent* |
| Ether | 5800 | 832 | 991 |
| Carbon disulfide | 1150 | 115 | 178 |
| Carbon tetrachloride | 1287 | 135 | 248 |
| Ethyl acetate | 750 | 108 | 148 |

* Calculated from Bliss' formula $Y=a+b(X-_x)$.

The chi-square test was applied to each of the calculated dosage-mortality curves in order to determine the homogeneity of the data. The chi-square calculated for the ether curve showed that more variation was present than is expected from random sampling of a homogeneous population. The chi-square test applied to the carbon disulfide curve (fig. 2) indicated strongly that these data are homogeneous and any variation present may be expected from random sampling. The data used in the determination of the carbon tetrachloride curve (fig. 3) show heterogeneity when analyzed by means of the chi-square test. The results of fumigation with ethyl acetate are homogeneous as shown by the chi-square test.

The presence of heterogeneity in the dosage-mortality curves for ether and carbon tetrachloride may be due to the fact that these two compounds cause a rather confusing paralysis making determination of the end point diffifficult in some of the insects exposed in the tests. As discussed in a previous section, ethyl acetate and carbon disulfide are more definite in action than are ether and carbon tetrachloride.

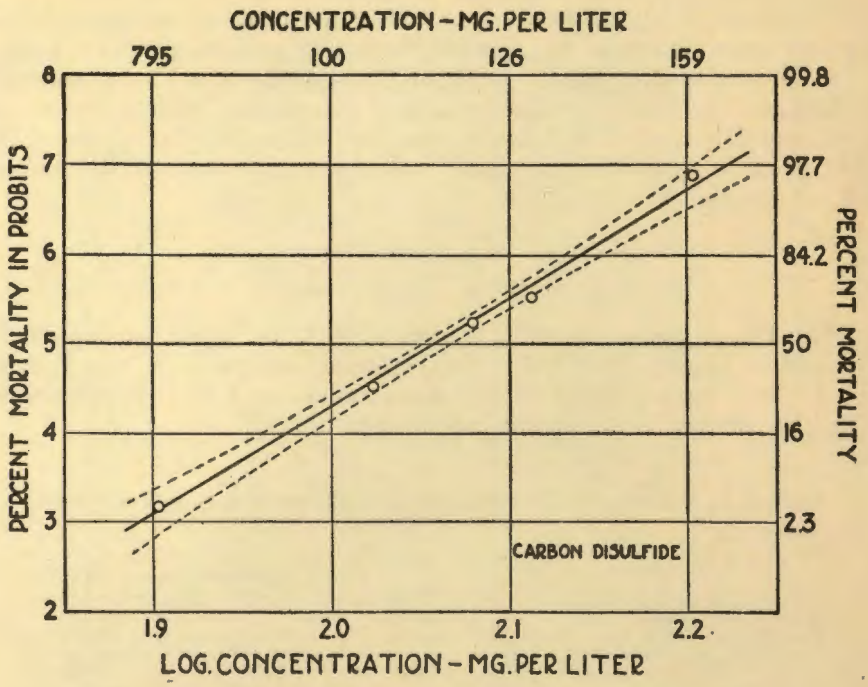


FIG. 2. Toxicity of carbon disulfide to *Tribolium confusum* Duval.

B. TOXICITY OF FUMIGANTS WITH SUBLETHAL CONCENTRATIONS OF ETHER

Following the experiments reported in the preceding section, it was thought desirable to determine the effect of sublethal concentrations of ether on the median lethal concentrations of the other gases.

The concentrations of ether to be used were determined in the following manner: small amounts of ether, ranging from 33 mg. per liter to 600 mg. per liter were placed in flasks containing flour beetles. Observations were made at frequent intervals and the concentrations used here were selected on the basis of the reaction of the insects. It was found that 33 mg. per liter did not anesthetize the beetles during the 2-hour exposure; 130 mg. per liter caused anesthesia in 45 to 60 minutes; and 520 mg. per liter anesthetized in less than 5 minutes after exposure. None of these concentrations killed any of the insects.

These concentrations (33, 130, 520 mg. per liter) were used with the approximate median lethal concentration of each of the other gases. It was thought that the primary effect of ether on the toxicity of the other gases might be due to its known anesthetic ability. If this were true there would be a consistent decrease in toxicity from 520 to 33 mg. per liter in the ether-fumigant mixture. The results of these tests are shown in table 2 and in figures 5, 6 and 7.

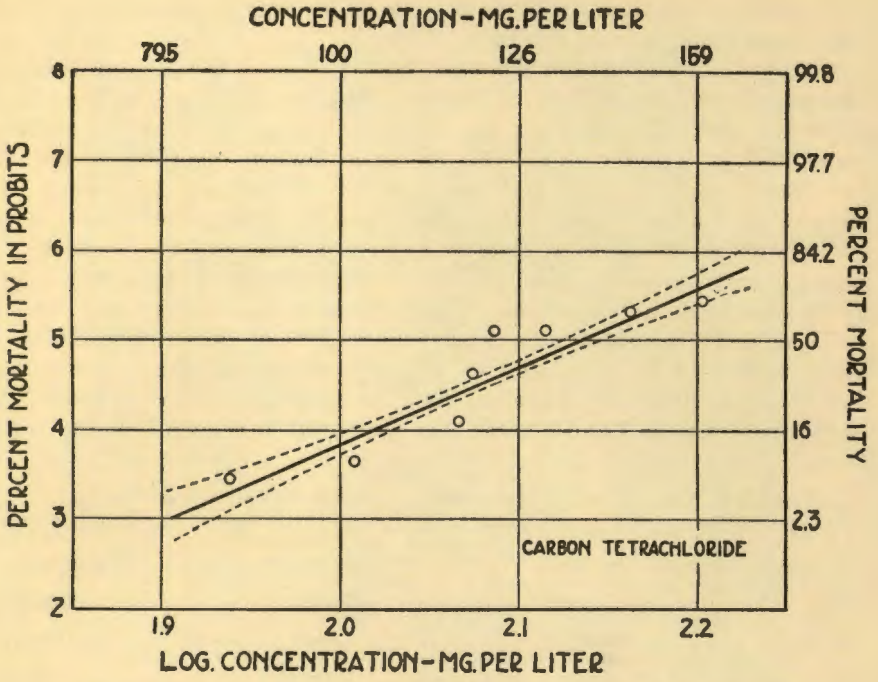


FIG. 3. Toxicity of carbon tetrachloride to *Tribolium confusum* Duval

The chi-square test as given by Snedecor (1937) was applied to these data. In table 2, the figure marked with an asterisk is the average of tests in which the variation was greater than that expected from random sampling. It is shown, however, that there is a definite trend through the regions of these points.

## DISCUSSION

The relative toxicity of ether, carbon disulfide, carbon tetrachloride and ethyl acetate to the confused flour beetle is shown in table 1. The median lethal concentrations are roughly in the ratio 7.7:1.0:1.2:1.0, indicating that carbon disulfide, carbon tetrachloride and ethyl acetate are all about seven times as toxic to *Tribolium confusum* as ether at an exposure of 2 hours at 30°C. It is interesting to note the rather small degree of variation at 50 per cent mortality shown by carbon disulfide, carbon tetrachloride and ethyl acetate, although these compounds differ widely from one another in chemical composition.

At the concentrations calculated to cause 99 per cent mortality, the relationship changes and the ratio ether: carbon disulfide: carbon tetrachloride: ethyl acetate is 6.7:1.2:1.7:1.0, indicating that at the higher level of mortality carbon disulfide, carbon tetrachloride and ethyl acetate

are, in general, less than six times as toxic to *Tribolium confusum* as ether under these experimental conditions.

Shepard, Lindgren and Thomas (1937) record the 5-hour median lethal concentrations at 30°C. for carbon disulfide (44 mg. per liter) and carbon tetrachloride (125 mg. per liter). It may be seen from these figures that at 5 hours and 30°C. the ratio carbon disulfide to carbon tetrachloride is 1:2.8, while at 2 hours and 30°C. this ratio is about 1:1.1. This seems to indicate that with carbon disulfide toxicity increases more rapidly with an increase in time of exposure than with an increase in temperature, whereas the toxicity of carbon tetrachloride increases more rapidly with a rise in temperature than with an increased exposure time.

These authors also include the concentrations of carbon disulfide and carbon tetrachloride calculated to kill 99 per cent of the test beetles. When the concentrations of carbon disulfide causing 50 per cent mortality at exposure times of 2 and 5 hours are compared, it is seen that the ratio

TABLE 2. *Toxicity of fumigants with sublethal concentrations of ether*

| Concentration in mg. per liter | | Expected Mortality Limits | Percentage Mortality Obtained |
|---|---|---|---|
| Ether | Carbon disulfide | | |
| ... | 115 | 45.5 to 53.9 | 50.0 |
| 33 | 115 | 45.5 to 53.9 | 82.2 |
| 131 | 115 | 45.5 to 53.9 | 97.3 |
| 522 | 115 | 45.5 to 53.9 | 100.0 |
| Ether | Carbon tetrachloride | | |
| ... | 130 | 41.1 to 47.2 | 44.0 |
| 33 | 130 | 41.1 to 47.2 | 88.7 |
| 130 | 131 | 41.1 to 47.2 | 97.8 |
| 521 | 130 | 41.1 to 47.2 | 95.3 |
| Ether | Ethyl acetate | | |
| ... | 107 | 43.7 to 56.3 | 50. |
| 33 | 107 | 43.7 to 56.3 | 71.2 |
| 130 | 107 | 43.7 to 56.3 | 56.5* |
| 522 | 107 | 43.7 to 56.3 | 41.5 |

5:2 hours exposure is 1:2.61 and at 99 per cent mortality, the ratio 5:2 hours is also 1:2.61. This would indicate that the curves for carbon disulfide at 5 hours and 2 hours at a temperature of 30°C. are nearly parallel throughout, since the ratios at the 50:99 per cent mortality are identical.

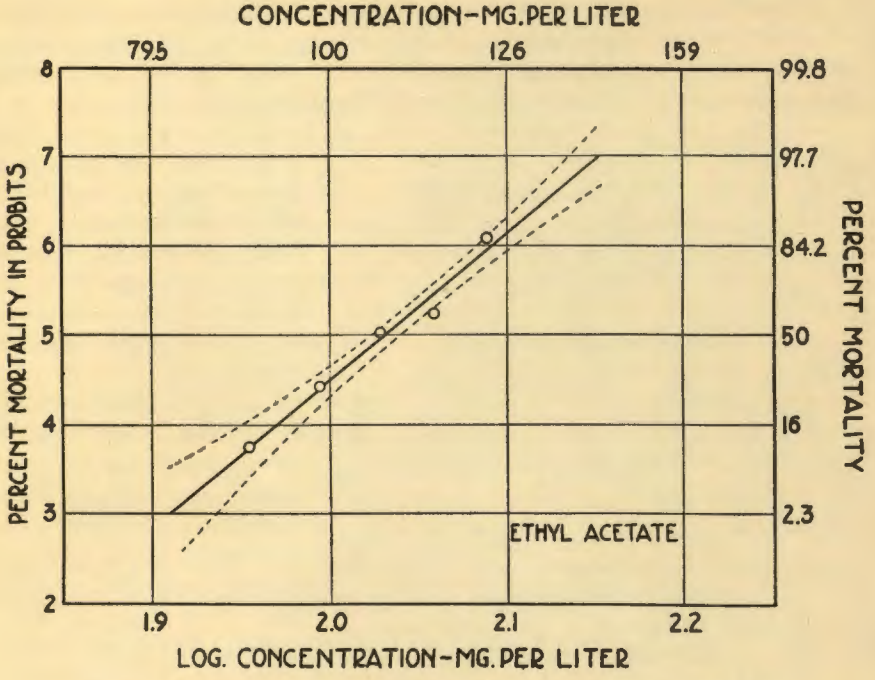


FIG. 4. Toxicity of ethyl acetate to *Tribolium confusum* Duval.

A comparison of the ratio 5:2 hours exposure of carbon tetrachloride at the 50 per cent point shows that it is about 1:1.08, while at the 99 per cent point the ratio 5:2 hours exposure is 1.97:1, indicating that the 5-hour dosage-mortality curve of carbon tetrachloride crosses the 2-hour curve at a point somewhere above the median lethal concentration.

This same phenomenon occurs in the carbon tetrachloride curves at 25°C and 30°C. where a 5-hour exposure was used, according to Shepard, Lindgren and Thomas (1937). The concentration of carbon tetrachloride killing 99 per cent at 25°C. is 405 mg. per liter, while that at 30°C. is 490 mg. per liter. It would appear that this effect of carbon tetrachloride is present over a wide range of temperature and exposures.

Mixtures containing the median lethal concentration of carbon disulfide, carbon tetrachloride or ethyl acetate with sublethal concentrations of ether show considerable differences in toxicity to the confused flour beetle. It is apparent from an inspection of figures 5 and 6 that carbon disulfide and carbon tetrachloride are similar in their actions when combined with sublethal concentrations of ether. Mixtures of both compound are least toxic to the confused flour beetle at an ether concentration of 33 mg. per liter. At higher ether concentrations the toxicity increases rapidly and approaches 100 per cent mortality. When the median lethal concentration of ethyl acetate is combined with sublethal concentrations of ether, the toxicity of the mixtures varies greatly. Figure 7

shows that the mixture containing 107 mg. per liter of ethyl acetate and 33 mg. per liter of ether is definitely synergistic, while mixtures containing greater amounts of ether show decreasing toxicity, and at an ether concentration of 521 mg. per liter, probable antagonism is shown.

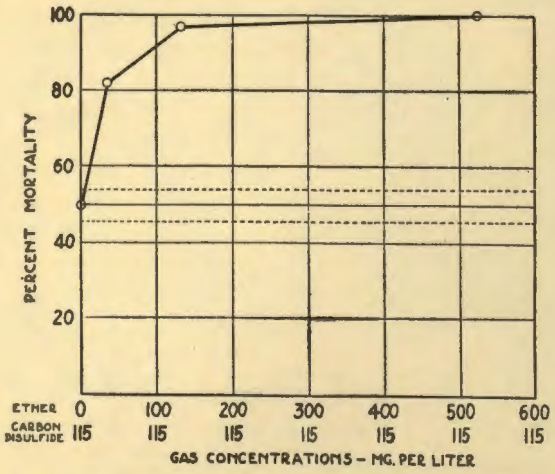


FIG. 5. Toxicity of carbon disulfide with sublethal concentrations of ether.

In a previous section, the action on the flour beetle of the sublethal ether concentrations used in these tests was described. Carbon disulfide appears to anesthetize or inactivate rather quickly insects exposed to this gas, while carbon tetrachloride inactivates test insects slowly and incompletely at low concentrations and more rapidly and completely at concentrations causing mortalities higher than 50 per cent. Ethyl acetate completely inactivates the confused flour beetle only at concentrations causing mortalities above 50 per cent.

These experiments indicate that carbon disulfide and carbon tetrachloride are more toxic to inactivated flour beetles, while ethyl acetate is more toxic (within limits) to active insects. It is possible that when sublethal concentrations of ether are added to the median lethal concentration of carbon disulfide or carbon tetrachloride, the ether vapor, being lighter and perhaps penetrating more rapidly than the vapor of the other compound, attacks the insects at once. This attack may be directed upon the cells of the nervous system or against some other system. Following this, the heavier gas may enter the body more freely

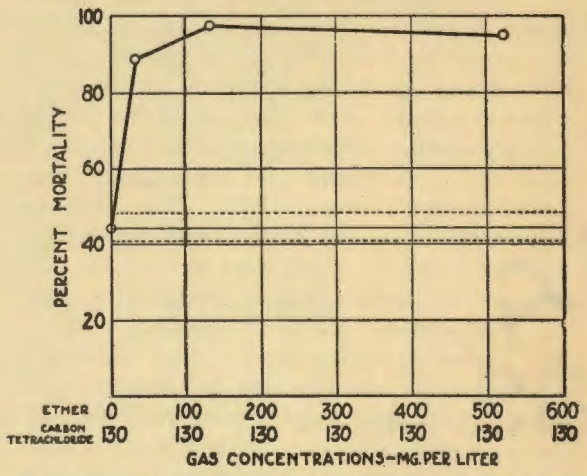


FIG. 6. Toxicity of carbon tetrachloride with sublethal concentrations of ether.

and might be actually allowed a longer period of exposure than when acting alone. It is suggested, however, that ethyl acetate may exert its toxic action to the greatest extent upon active beetles, possibly because of poorer penerating ability, and that anesthetizing sublethal concentrations of ether cause a "protective stupefaction." The existence of this condition was first brought out by Gray and Kirkpatrick (1929) in experiments on HCN-resistant black and red scales.

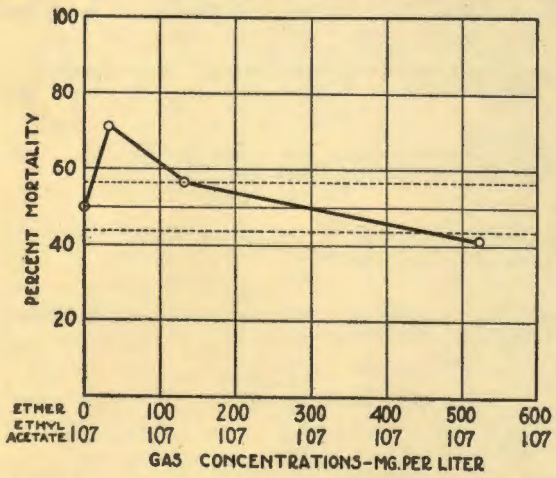


FIG. 7. Toxicity of ethyl acetate with sublethal concentrations of ether.

## SUMMARY AND CONCLUSIONS

The 2-hour dosage-mortality curves of ether, carbon disulfide, carbon tetrachloride and ethyl acetate at 30° C. against the confused flour beetle (*Tribolium confusum* Duval) are presented. Carbon disulfide, carbon tetrachloride and ethyl acetate are more than seven times as toxic to the flour beetle as ether at the median lethal concentration under these experimental conditions.

The effect of certain sublethal concentrations of ether upon the toxicity of the median lethal concentrations of carbon disulfide, carbon tetrachloride and ethyl acetate was studied. It is shown that the toxicity of carbon disulfide and carbon tetrachloride is increased as the concentration of ether increases. The toxicity of ethyl acetate to the confused flour beetle decreases rapidly as the concentration of ether increases, and it was found that the highest sublethal concentration of ether used was apparently antagonistic with ethyl acetate.

Sublethal concentrations of ether increase the toxicity of carbon tetrachloride and carbon disulfide in proportion to the concentration of ether present. Sublethal concentrations of ether decrease the toxicity of ethyl acetate in proportion to the concentration of ether in the mixture.

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